ABSTRACT

Sharon Nappier: The Use of Thermal Inactivation of Male-Specific Coliphages as Potential Indicators of Pathogen Reductions in Thermophilic Anaerobic Digestion
(under the direction of Dr. Mark Sobsey)

Male-specific (F+) coliphages were investigated as indicators of virus inactivation during thermophilic digestion and other thermal processes. Because pathogen monitoring in sludge is difficult, costly, and impractical, microbial indicators are needed to predict pathogen reductions in Class A biosolids. F+ coliphages are a promising candidate indicator of fecal contamination and a surrogate for virus reduction in waste treatment processes. F+ RNA group I, F+ RNA group III, and F+ DNA phages were isolated from municipal sludge. In laboratory studies the kinetics and extent of inactivation of groups I and III F+ RNA, and F+ DNA coliphages were quantified and compared at 53 °C, a temperature used in thermophilic anaerobic digestion. Isolates of groups I and III F+ RNA and F+ DNA coliphages were exposed to 53 °C in an automated, thermoregulated heat block and titered after 0, 5 and 60 minutes. After 60 minutes at 53 °C, F+ RNA group III coliphages were inactivated significantly greater ($P < .0001$) than the F+ RNA group I and F+ DNA coliphages. The inactivation kinetics of group III F+ RNA coliphages resemble those of some enteric viruses and some other pathogens. The results of this study elucidate the thermal properties of the male-specific coliphage subgroups and facilitate the development of time and temperature relationships that predict enteric virus and other pathogen reductions in thermal and thermophilic processes used to meet Class A biosolids requirements.

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LIST OF ABBREVIATIONS

ANOVA = analysis of variance
BOD = biological oxygen demand
bp = base pairs
C = Celsius
$CBFT^3$ = Columbus Biosolids Flow Through Thermophilic Treatment
CFR = Code of Federal Regulations
CFU = colony forming unit
DNA = dioxyribonucleic acid
E. coli = *Escherichia coli*
EPA = Environmental Protection Agency
F+ = male-specific coliphage
F+ DNA = male-specific DNA coliphage
F+ RNA = male-specific RNA coliphage
FC= fecal coliforms
g = grams
ml = milliliters
MPN = most probable number
NH_3 = ammonia
NR = no removal
OWASA = Orange Water and Sewer Authority
PBS = phosphate buffer solution
PCR = polymerase chain reaction
PFRP = processes to further reduce pathogens
PFU = plaque forming unit
POTW = Publicly Owned Treatment Works
RLB = reverse line blot
RNA = ribonucleic acid
RPM = rotations per minute
RT-PCR = reverse transcriptase-polymerase chain reaction
SCWRF = South Columbus (GA) Water Resources Facility
SS = suspended solids
ss-DNA = single stranded DNA
ss-RNA = single stranded RNA
TAD = thermophilic anaerobic digestion
TNTC = too numerous to count
TS = total solids
TSB = tryptic soy broth
WSSLD = Western Lake Superior Sanitary District
µl = microliters

1 INTRODUCTION

In the United States, the first comprehensive sanitary sewer system was not constructed until 1855 (in Chicago) and the first sewage treatment facility in 1886 (New York City). Currently there are over 19,400 municipal Publicly Owned Treatment Works (POTWs) in the U.S., treating roughly 50 billion m^3 of raw sewage a year (NAS, 1996). As the sewage is treated, it goes through a series of processes that reduce the concentration of the easily-decomposable organic materials and pathogenic organisms. Sewage sludge is the material before it receives biological or chemical treatment and biosolids are the generally settleable solid biological residual produced during the treatment of sewage wastewater. It is often a mixture of human and industrial wastes and may consist of no more than 1 % of the wastewater flow. Characteristics of the sludge depend on the wastewater treatment process and sludge treatment (Eberle et al. 1994).

The installation and operation of wastewater treatment facilities contribute significantly to pollution reduction, prevention of damage to the environment, and protection of public health. However, the discharge of sewage sludge, whether treated or not, can create serious water pollution problems in the receiving body of water or land. Raw sewage often contains many pathogenic organisms, which is a great potential public health concern. Additionally, the high concentrations of suspended solids (SS) and nutrients, as well as the organic matter (e.g., BOD) of raw sewage, may create eutrophication problems. The processing and final disposal of sludge is a challenging and costly task. The three most

common methods for final disposal of sludge are landfills, land application, and incineration (Zhou and Mavinic 2003).

Sludge incineration fires sludge at high temperatures in a combustor. Incineration is costly and energy intensive. It produces off-gas that needs to be regulated (e.g. hydrocarbons, and carbon monoxide). Sludge disposal to landfills, mostly disposed along with municipal solid wastes, results in difficulties in compression of disposed materials. The capacities of existing landfills are diminishing; however, the amounts of sludge and disposal costs continue to increase. It is more and more difficult to find appropriate and adequate new sites for landfills (Zhou and Mavinic 2003).

It is recognized that beneficial land application (e.g. agricultural soil conditioning or forest fertilizing) of processed sludge (biosolids) is a cost-effective and environmentally sustainable option, because nutrients and organic matter are recycled to soils. In 1993 the USEPA released the technical standards (40 CFR Part 503) for the use and disposal of biosolids, and introduced the criteria for Class-A biosolids. Class-A biosolids are essentially pathogen free, meet acceptably low metal concentrations and vector attraction reduction requirements, and can be beneficially land-applied without restrictions (US EPA 1999; NAS 2002; Zhou and Mavinic 2003). These requirements are met through Processes to Further Reduce Pathogens (PFRPs), which transform sludge from waste pollutants with high levels of pathogens into a valuable biosolids resource (Zhou and Mavinic 2003).

PFRPs that are currently recognized by the EPA are composting, heat drying, heat treatment, thermophilic aerobic digestion, beta ray irradiation, gamma ray irradiation, and pasteurization. When these processes are operated using specified conditions, pathogenic bacteria, enteric viruses, and viable helminthes ova are reduced to below detectable levels

(US EPA 1999; NAS 2002). Despite the potential for thermophilic anaerobic digestion to meet Class A criteria, this process is not currently recognized as a PFRP to produce Class A Biosolids.

In addition to the pathogen requirements, Class A biosolids must be monitored for indicator organisms, fecal coliform, or *Salmonella* spp. bacteria (1) at the time of use or disposal; (2) at the time the biosolids are prepared for sale or given away in a bag or other container for land application; or (3) at the time the biosolids are prepared to meet three requirements for "exceptional quality" sludge to ensure that growth of bacteria has not occurred (US EPA 1999). Municipalities and POTWs typically rely on assays of indicator microorganisms to detect pathogen reductions or excessive microbial contamination of public health concern.

To protect the public's health and to save time and money, it is impractical to directly monitor, identify, or enumerate the hundreds of pathogenic microorganisms associated with fecal pollution. Over 140 viral pathogens have been identified from human feces, new ones continue to be discovered and it is believed that there are many others still unknown (Gantzer et al 1998). While methods have been developed for detecting and assaying specific viruses or virus groups, they are often expensive, technically demanding, and time-consuming. Also, viruses are often present in low but still risky concentrations, which may require the analysis of large sample volumes, such as more than ten liters of water or more than 100 ml of sludge. The use of indicators entails quantifying more plentiful microbes that commonly inhabit the intestines of warm-blooded animals and considering them a sign that more hazardous pathogenic organisms may also be present (Gerba 1987; Sobsey 1995).

Currently fecal coliform bacteria are the standard microbial indicator used in the United States for biosolids and municipal waste water. However, because viruses are smaller and are more resistant to inactivation processes than bacteria, fecal coliforms are considered inadequate indicators of waterborne enteric viruses (such as Hepatitis A virus, *Rotaviruses,* and *Adenoviruses*, and small, round structured "gastroenteritis viruses" such as *Astroviruses* and *Noroviruses*, including Norwalk virus) (Havelaar 1993).

Enteric bacteriophages, viruses infecting enteric bacteria, have been proposed as more reliable indicators for human viral pathogens (Gerba 1987; Leclerc et al. 2000). Coliphages, viruses which infect *Escherichia coli*, have probable fecal origins, are consistently found in sewage, and are also easily, quickly, and inexpensively quantifiable.

There are several different taxonomic groups of enteric bacteriophages, not all of which may be good virus indicators. Bacteriophages that infect their hosts through receptors on the cell wall are "somatic." "Male-specific" coliphages infect their hosts through receptors of F pili, which are surface appendages encoded by F-plasmid genes. The somatic coliphage families include *Myoviridae*, *Microviridae*, *Siphoviridae* (or *Styloviridae*) and *Podoviridae*. *Leviviridae* and *Inoviridae* are male-specific coliphage families which contain RNA and DNA genomes, respectively. F+ RNA coliphages can be subdivided further into four groups: group I (MS2), group II (GA), group III (Qβ), and group IV (SP). Different persistence of phages in the environment is suggested to be related to phage morphology (Lasobras et al. 1997). It is thought that the physical structure, composition and morphology of F+RNA phages closely resemble those of human enteric viruses. The extent to which coliphages are reliable indicators of pathogen reductions by sludge treatment processes such a thermophilic digestion has not been determined.

General Objectives

This study examined male-specific (F+) coliphage inactivation in a lab-scale thermophilic anaerobic digester and the potential use of these phages as an indicator of pathogen reduction in thermal processes to meet Class A biosolid requirements. Because of observed bi-phasic inactivation kinetics in thermophilic anaerobic digestion studies (Aitken et al. 2003), F+ coliphages in raw and treated sludges were further characterized as either F+ DNA or F+ RNA, and the F+ RNA coliphages were further identified and characterized as to which group they belonged. Groups I and III F+ RNA and F+ DNA coliphages were isolated and characterized from the untreated sludge (feed), thermophilically digested sludge from a continuous flow reactor, and from the biosolids exposed to a thermophilic temperature for various periods of time in the batch reactor. Because of evidence that these coliphages may possess different heat sensitivities, the isolates were further evaluated for inactivation kinetics in a lab-scale heat exposure experiment at 53 °C for 5 minutes and 60 minutes.

Specific Objectives

1. Evaluate the inactivation of male-specific coliphages by thermophilic anaerobic digestion (TAD) in continuous flow and batch reactors at 51, 53, and 55 °C.
2. Determine the inactivation kinetics of male-specific coliphages at thermophilic temperatures in batch experiments.
3. Isolate infectious male-specific coliphages from untreated sludge and treated biosolids from a laboratory thermophilic anaerobic digester.

4. Genotype and/or serotype F+ DNA isolates as Fd, M13, or CH and F+ RNA isolates as group I, II, III, or IV.
4. Evaluate F+ RNA and F+ DNA coliphage sensitivity to 53 °C for 5 minutes and 60 minutes.
5. Characterize the identities of the heat-resistant and heat-sensitive male-specific bacteriophage fractions occurring in inactivation rate experiments in the digested biosolids.
6. Determine if particular groups or types of F+ RNA or F+ DNA coliphages could serve as useful indicators of pathogen reductions in Processes to Further Reduce Pathogens (PFRP) based on their inactivation rates in thermophilic anaerobic digestion and thermal inactivation experiments.

II REVIEW OF THE LITERATURE

Primary and Secondary Treatment and the Creation of Sludge

When raw sewage first arrives at the POTW, it usually passes through a screening device, consisting of a series of metal bars, to remove large objects that have entered the sewer pipes and might damage components of the POTW. The sewage then flows into a grit chamber, where particles roughly the size and density of sand grains settle out. Removal of this coarse grit is intended to protect the pumps in the rest of the system. With large objects and grit thus removed, the sewage is pumped to a primary settling tank or primary clarifier. In a typical treatment plant, much of the flow through the rest of the system is by gravity (NAS 1996; Laws 2000).

In the primary settling tank, settleable or floatable solids are removed from the sewage during a period of about 3 hours. The tank may be circular or rectangular. In a circular tank, the sewage enters at the center and slowly flows to the edge where it exits under a baffle. In a rectangle tank, the sewage enters at one end and exits at the other. Solid materials that float to the surface during the residence time of the sewage in the tank are removed by a mechanical skimming device that pushes floating objects to a hopper, from which they are pumped into the anaerobic digester. The bottom of the tank is inclined, and a similar mechanical device moves the sludge that accumulates on the bottom toward the deepest part of the tank, where it is drawn off and usually is pumped to the anaerobic digester. The material skimmed from the surface and from the bottom of the primary tank is called the primary sludge. Treatment involving no more than removal of this primary sludge

is termed primary sewage treatment. It is required by federal law that a POTW operating with primary treatment only, must remove at least 30% of the BOD and SS from the sewage (Laws 2000).

Phage removal is fairly low and erratic during the screening, grit chamber, and primary sedimentation processes. Here, most viruses in the sewage are reduced by settling with only 0-30% efficiency. The majority of the viruses remain in the primary effluent, but those entering the sludge cause the sludge to have a higher virus concentration than the raw sewage. In one experiment in which phage f2 was seeded into the wastewater the mean phage reduction was 37.1% (27.8-54.2%) in 2.5 million gallons/day by primary treatment (Bitton 1987).

If secondary treatment is used, the effluent from the primary tank flows into a second tank where biological processes are used to remove much of the BOD in the effluent. Here, organisms in the tank consume the organic substances in the primary effluent. In this process, the consumer organisms grow and multiply, while large percentages of the organic substances are respired. As a result, the amount of potentially oxidizable material in the waste is greatly reduced. The effluent from this treatment process consists in part of the organisms that have been feeding off the organic wastes. Most of the biomass of these organisms can be removed by transferring the effluent into a secondary settling tank or clarifier. There the biomass settles to the bottom in the form of a floc and is removed in a manner similar or identical to that employed in the primary tank. A portion of the secondary sludge is then removed for separate treatment and disposal, while the remainder is recycled (Laws 2000).

In the activated sludge process, phages may be removed through: adsorption to or embedding within flocs, inactivation by sewage bacteria, or ingestion by protozoa. The secondary treatment step efficiently inactivates phages, normally showing greater than 90% reduction of infectivity. One study of phage removal through activated sludge found the efficiency range between 96-97.3%, while the enteroviruses averaged 92% removal efficiency (Bitton 1987). Additionally, when phages and *E. coli* samples were monitored weekly for a one-year period, it was discovered that phages were removed through activated sludge processes efficiently and were better gauges of enteroviruses than was *E. coli*.

Thermophilic Anaerobic Digestion

Anaerobic digestion is among the most common methods of treating primary sludge and the waste biomass from biological treatment processes such as activated sludge. The anaerobic digester is a closed, usually cylindrical tank, where it provides food for a special class of microorganisms that carry on their metabolic activities in the absence of oxygen, i.e. anaerobically. The products of the anaerobic digester are digested sludge (biosolids), a supernatant fluid, and gases. The digested sludge is relatively stable and inoffensive compared to the undigested primary and secondary sludges. Furthermore, its volume is only about one-third that of the primary and secondary sludges. This reduction in volume is achieved both by the conversion of some of the organics in the raw sludge to gases, (primarily methane and carbon dioxide), by anaerobic catabolism, and by dewatering the sludge to reduce its moisture content. The raw sludge typically has a moisture content of 94-99% by weight (1-6 percent solids), and most of its volume is due to water (Laws 2000).

The anaerobic digester is invariably heated to speed up the metabolic rate of the microbes. For mesophilic anaerobic digestion, operating temperatures are usually in the range of 27-35 °C. For thermophilic anaerobic digestion, operation temperatures are between 50 and 70 °C, although usually the maximum temperature is no more than 60 °C (Zhou and Mavinic 2003). The methane gas produced in the digester is adequate to provide fuel to heat the digester to the desired temperature. The excess methane produced in the digester is burned either as a waste product or often coupled to the production of electricity. The supernatant fluid from the digester retains some undesirable and objectionable properties (high organic matter and odor) and is usually recycled through the secondary treatment process. The only product of the digester that requires disposal is the stabilized sludge.

In 1993, US EPA launched the need for special disinfection of biosolids such as digested sewage sludge before it can be deposited on agricultural lands or have other less restrictive disposal options (US EPA 1999). Mesophilic digested biosolids are classified as Class B quality and have very restricted use. Sludge digestion at thermophilic temperatures or by other advanced processes is necessary to meet new standards for Class A Biosolids that allow for land application and other less restrictive disposal options.

Thermophilic digestion can be carried out either anaerobically, or aerobically. Larger treatment facilities tend to operate anaerobically (e.g. Greater Vancouver Annacis Island Wastewater Treatment Plant, 450 ML/day flow) (Krugal et al. 1998; Zabranska et al. 2003). Smaller or medium size facilities tend to operate aerobically (e.g. British Columbia's Whistler Wastewater Treatment Plant, 12.5 ML/day flow) (Zhou and Mavinic 2003). The advantage of the anaerobic digestion of sludge is production of energy as biogas (Zabranska et al. 2003). The produced energy can not only supply the energy needed for the sludge

treatment but also provide a substantial part of the energy used by the whole treatment plant. The thermophilic temperature range of the anaerobic process brings a higher efficacy in the degradation of organic matter associated with a higher biogas production and a lower content of volatile solids in the digested sludge, which represents a lesser amount of output stabilized sludge with better hygienic quality (Zabranska et al. 2003).

The operational temperature of thermophilic anaerobic digestion (TAD) is usually 55 °C. Important reasons for applying thermophilic temperatures are the better sanitizing effect of the higher process temperature and the ability to use lower retention times than used for mesophilic digestion. By upgrading existing mesophilic digesters to the thermophilic temperature range, the digesting capacity can be doubled and considerable reduction of pathogen content can be obtained (Krugal et al. 1998; Zabranska et al. 2003, Zabranska et al. 2003). For practical operation of full-scale plants, a temperature between 52 and 56 °C is preferable, allowing a slight variation in the temperature without fatal consequences for some of the active microbes (Ahring 1994). These thermophilic temperatures kill most pathogenic bacteria and most viruses. However, some heat-resistant viruses such as the parvovirus will not be killed at a digestion temperature of 55 °C (Lund et al. 1996 and Borovec et al. 1998). Therefore, increased demands for pathogen kill during anaerobic digestion of sludge could increase the interest for digestion at higher temperatures of 60-65 °C to ensure that the material is properly sanitized for allowing unrestricted use as a fertilizer on farmland (Ahring 2001).

In an evaluation of a TAD at Lions Gate in Vancouver, they successfully transitioned from a mesophilic to thermophilic process. They found that a two-stage thermophilic process, operating at 55 °C, consistently produced biosolids meeting the Class A fecal

coliform (FC) requirements with non-detectable levels of *Salmonella* bacteria. After the first stage, measurements average slightly above Cass A levels of FC of 1000 MPN/g TS and after the second stage, FC measurements are reliably below the fecal coliform upper limit for Class A requirements. Volatile solids destruction and methane gas production were slightly higher than in the previous mesophilic operating mode, and operators reported that thermophilic biosolids are easier to dewater and handle. The visual appeal of the thermophilic biosolids as an agricultural amendment is also much improved, having a light granular texture (Krugal et al. 1998).

The stable and efficient operation of the thermophilic anaerobic digester strongly depends on the activity of anaerobic consortia in the digester (Zabranska et al. 2003). There is a lack of understanding of the differences between microorganisms active at the thermophilic temperatures compared to mesophilic temperatures. Sewage sludge or other types of waste will mainly house mesophilic microbes including anaerobes (Ahring et al. 2002). However, the numbers of thermophiles and especially some specific groups such as acetate-utilizing methanogens, propionate-degraders or cellulose-degraders will often be very low or these groups can even be missing in the waste (Ahring 1994). The occurrence of thermophiles will depend upon the history of the waste material and therefore some material will sometimes have unpredictably high numbers of, for instance, thermophilic methanogens (Ahring 2002).

Class A Biosolids and Land Application of Sewage Sludge

As stated above, there is considerable motivation for applying sludge to land. The EPA first promulgated criteria for land application of sewage sludge to cropland in 1979, and current standards for the use and disposal of sewage sludge were instituted in 1993 (US EPA

1999), which are commonly referred to as the "Part 503 Sludge Rule." This rule puts sludge into one of two categories: Class A and Class B. Class A sludge is safe for direct contact and can be used in an unrestricted manner. However, Class B sludge is of lesser quality and its use is regulated with more stringent standards pertaining to the limitations of disposal options and restrictions on access or content. The general criteria for pathogens and fecal coliforms in Class A biosolids are summarized in Table 1.

Table 1. Pathogen and Indicator Criteria for Class A Biosolids

Item	Criterion
Viable helminthes ova	< 1 per 4 g dry solids
Enteric viruses	< 1 per 4 g dry solids
Salmonella species	< 3 cfu per 4 g dry solids
Fecal coliform	< 1,000 per g solids

Because sludge is a potential source of nutrients and a good soil conditioner, land application of sewage sludge is a very practical and desirable means of disposal. Currently about 60% of the municipal sewage sludge in the U.S. is now land applied (NAS 2002). One important consideration in the land application of sludge is the fact that sludge can be transported economically much greater distances than wastewater. For example, New York City's treated sludge is currently shipped to northeastern Texas and eastern Colorado for cropland application. Another consideration is that the use of sewage sludge has been very successful in the restoration of strip-mined land. Establishing vegetation on such land is extremely difficult due to the lack of nutrients, low organic matter content, low pH, low water retention and toxic levels of metals in the soil (NAS 1996). Sewage sludge overcomes these difficulties. Given the present and future anticipated extent of strip mining activity, it

seems likely that a large percentage of the sludge generated in the U.S. could be used for this purpose for many years.

Crop yields on land fertilized with sludge often exceed those obtained with commercial fertilizer (NAS 1996). Therefore, sewage sludge has considerable potential to provide agronomic benefits with out the use or with reduced use of commercial fertilizer. However, there are concerns that pathogens, nitrate, and heavy metals, such as lead and cadmium, will contaminate groundwater or that pathogens will be transferred to humans or grazing animals that ingest plants with sludge-contaminated surfaces (Laws 2000). Therefore, the extent to which treated sludge contains pathogens when land applied and the analysis and management of these risks is needed in order to reduce them to acceptable levels.

Potential Effects of Pathogens

Existing data indicate that pathogenic organisms (bacteria, viruses, or parasites) occur in sewage sludges and, for varying time periods, in sludge-amended soils. There is a lack of evidence of disease outbreaks from bacteria and viruses due to the application of secondary, activated, anaerobically digested, or chemically treated sewage waste (Kelley et al., 1984; NAS 2002). These organisms tend to lose virulence or become inactivated after treatment by these processes and within the soil environment as a result of exposure to such factors, as temperature, sunlight, soil pH, moisture, the availability of organic matter, soil particles, and the presence of toxic substances and competitive organisms. According to some earlier analyses of the issue, sludge-borne bacteria and viruses are not a serious threat to health because these pathogens are poor competitors outside the host (Kelley et al. 1984). However, more recent analyses support previous recommendations that precautionary

measures, especially by persons directly involved in applying the sludge to land (NAS 2002), are needed to prevent a serious outbreak of disease in the future. It has also been suggested that wastes should not be applied to food crops during the current growing season or to above-ground corps for 1 month before harvest. Additionally, animals should not be allowed to graze in an area for 2 to 3 weeks after waste application (Kelley et al. 1984). The factors that influence the risks of pathogens in sludge to be land applied include the types and properties of the pathogens themselves. Therefore, the types of pathogens and their properties such as survival under different environmental conditions must be understood.

Helminthes

The principle concern with the land application of biosolids is the presence of parasite ova and cysts, which tend to become concentrated in the sludge during the process of sewage treatment. Helminth ova are resistant to the environmental factors and wastewater treatment processes that often reduce the concentration of bacteria and viruses in sludge. The potential for parasitic disease transmission by land disposal of sewage sludge is important because the environmental forms of these organisms are extremely resistant and can persist and remain infective for long periods in the soil (NAS 1996; NAS 2002). As seen in the table above, the concentrations of such ova is one of the principle criteria for determining whether sludge is classified as Class A or Class B.

Helminthes of greatest concern produce infectious cysts or eggs that require a period of development in the external environment (a soil-transmitted parasite or a non specific intermediate host) and a simple fecal-oral transmission pathway. Cysts and eggs produced by parasitic species are quite resistant to destruction by adverse environmental conditions

(NAS 1996) and sewage treatment processes such as disinfection with chemicals such as chlorine and ozone. Additionally, secondary treatment processes, such as trickling filtration, intermittent sand filtration, and activated sludge, will reduce the numbers, but will not completely destroy these parasitic forms (Kelley et al. 1984; Hays 1977). The most important factor affecting the survival of all helminth eggs is temperature, with rapid inactivation resulting from temperatures below freezing and above 45 °C for at least some types of them (Brown & Root Services 2001). Studies have shown that irradiation or maintenance of 60 °C temperature for 30 minutes (Kelley et al. 1984) is necessary to ensure destruction of cysts and eggs of all types (Hays 1977). The more thermally resistant organisms such as *Cryptosporidium* oocysts and *Ascaris* ova have much longer survival times than some other parasites and consequently provide a risk of infection if not adequately treated (Brown & Root Services 2001).

Bacteria

Bacteria are often inactivated by high temperatures. However, different bacteria are influenced differently by elevated temperatures and the effect depends on the temperature itself. Most of the enteric bacteria of concern in sludge are capable of being inactivated at thermophilic temperatures. However, some bacteria can produce spores that are extremely resistant to environmental stresses. Other bacteria may produce dormant states where metabolic activity is decreased and cell size is reduced to aid survival under conditions of starvation (Brown & Root Services 2001). Additionally the presence of other microorganisms in the environment affects the survival of bacteria through predation, competition for nutrients and as a source of nutrients.

Protozoa

The most common enteric protozoan parasites associated with biosolids from wastewater treatment are *Cryptosporidium* and *Giardia*. They have been observed to die within days of Class B biosolid treatment, but there is still little research on the survival of these parasites in biosolid-amended soils (NAS 2002). Gerba et al. (2002) noted that they are unlikely to survive mesophilic digestion temperatures or the drying that occurs after land disposal.

Viruses

Next to helminthes, enteric viruses may present the greatest risk because of the resistance to inactivation by heat by some of them. Most food or water-borne viruses are more resistant to heat, disinfection, and pH changes than are most vegetative bacteria. Numerous studies have addressed the stability of viruses under different conditions, but little has been done to standardize or systematically compare these studies. However, it has been noted that one of the most important parameters controlling virus persistence in the environment is temperature.

The virus type, structure, and composition often influence disinfection efficacy and inactivation. For example, glycoproteins, typically on the outer surface and the outer envelopes of enveloped viruses are often labile to disinfectants. The environment (or medium) in which viruses are found also influences their sensitivity to thermal inactivation (Croci 1999; Koopmans and Duizer 2003). A protein environment protects the viruses from the action of heat. Additionally virus survival can be increased if they are embedded within other material (within a membrane), through aggregation with other virus particles, and through adsorption to organic or soil particles where they are protected from environmental

influences (Brown & Root Services 2001). Table 2 is a review of the various virus inactivation temperatures. It strongly stresses the need for virus specific studies to address virus inactivation. However, it is also important to recognize that virus inactivation by heat is dependent on both temperature and contact time, as will be discussed in the next section below.

Table 2. Enteric Virus Thermal Inactivation Chart

Virus	Temperature/Time	Activity	Citation
Enterovirus	42 °C (most enteroviruses)	Inactivated	Fields et al. 1996
Poliovirus	85-90 °C / 1 min (in cockle meat)	Inactivated	Slomka and Appleton 1998
Hepatitis A Virus	60 °C / 60 min	Stable	Fields 1996
	85-90 °C / 1 min (in cockle meat)	Inactivated	Slomka and Appleton 1998
	70 °C / 4 min, 75 °C / 30sec, 80 °C / 5 sec, 85 °C/ instantly	Inactivated	Borovec 1998
Rhinovirus	60 °C / 60 min	Labile	Fields et al. 1996
type 3-12, 15,18,19	50 °C / 60min	Stable	Fields 1996
type 14,16,17	50 °C/ 60 min	Partially Inactivated	Fields 1996
type 13	50 °C / 60 min	Inactivated	Fields 1996
Calicivirus			
Norovirus	60 °C/30 min	Retains Infectivity	Fields 1996
	100 °C	Inactivated	Koopmans and Duizer 2002
Astrovirus	60 °C/10 min	Inactivated	Bock and Whelan 1987

Virus Inactivation Kinetics

In addition to the inactivation temperatures of viruses, there is also a considerable role for contact time. This is because the inactivation of viruses and other microbes is a kinetic process that can be described in terms of the extent of inactivation per unit of time at a given exposure temperature. It is often assumed that viruses and other microbes are inactivated at a constant rate at a given temperature; that is inactivation kinetics are first-order or

exponential. However, there has been a considerable amount of discussion over the significance of variations in survival curves of microorganisms subjected to the lethal action of physical agents (in this case, heat). For example, it is imperative for scientists to understand and describe the relationship between the number of organisms surviving and the duration of treatment with the disinfecting agent. First-order kinetics was first described by Chick's Law to illustrate the rate of reaction is directly proportional to the concentration of the reacting substance. It assumes all organisms are identical and the inactivation results from a first order or exponential reaction. This first-order relationship is described mathematically below.

First Order Kinetics in a batch system:

$$dN/dT = -kN$$

where: N = number (concentration) of organisms
T= time
k= is the proportionality constant
dN/dT = the rate of change of concentration with time

The solution to this equation is :

$$N/N_o = e^{-kt}$$

No = initial number of organisms at time T=0
Nt = number of organisms remaining at time = T

Also:

$$\ln N_t/N_o = -kT$$

Figure 1. First-Order Kinetics

In most general terms, this principle states that the survival ratio is a decreasing (negative slope) exponential function of time (e^{-kt}), from which it follows that the logarithm of the survival ratio is a straight line when plotted against time. The relationship indicates that each infectious unit has the same fixed probability of being inactivated per unit time and

implies that (i) the virus preparation is homogeneous, and (ii) inactivation does not require cumulative damage (Hiatt 1964; Sobsey 2003).

However, departures from first-order kinetics are common. It was originally postulated that the deviations from the exponential law resulted from heterogeneity of the microorganisms, or from mechanistic factors, which attributed these deviations to factors operating during the reaction (Hiatt 1964). The other types of curves that have been seen are known as retardant curves ("tailing effect" or declining inactivation kinetics over time) and shoulder curves ("multihit kinetics" or an initial slow rate of inactivation, followed by a faster rate) (Sobsey 2003; Maier 2000).

The retardant curve can be illustrated as a "tailing effect" or decrease in inactivation rate as time is extended. The change in slope may be abrupt or gradual. Tailing effects which do not occur until the survival ratio is less than 10^{-5} or 10^{-6} are likely to be difficult or impossible to explain, for there are many indeterminate factors which might extend the longevity of one or more virus particles out of each 100,000 or million originally present. It has often been attributed to physical factors such as aggregation, adsorption to walls of the vessel, or presence in aerosol droplets above the surface of the liquid, which might protect against the inactivating process and are appreciable if their probability of occurrence is 10^{-5} or $^{-6}$ or greater. However, it has been postulated that infectious particles may develop a resistance to the inactivating process as the reaction proceeds (Hiatt 1964).

Further, the shape of the curve may indicate an inherent heterogeneity of the virus or more than one population of virus present. For example, in heat inactivation experiments, there may be a small proportion of thermally resistant particles to account for the decreased slope of the latter segments of these curves. Below is a two-population model that may be

used to describe the results of heat inactivation experiments that illustrate heat-sensitive or heat-resistant fractions of the organisms (Hiatt 1964; Aitken et al. submitted).

A two population model may appear as follows:

$$C_{total} = C_R + C_S$$

C_R = the total concentration of resistant organisms
$C_{R,O}$ = the initial concentration of resistant organisms

C_S = the concentration of sensitive organisms
$C_{S,O}$ = the initial concentration of sensitive organisms

If, each population decay is by first-order kinetics:

$$C_R = C_{R,O}(e^{-k1t}) \text{ and } C_S = C_{S,O}(e^{-k2t})$$

And:

$$C_{total} = C_{R,O}(e^{-k1t}) + C_{S,O}(e^{-k2t})$$

If k_1 is assumed d= 0 (i.e. No detectable decay of resistant organisms), then:

$$C_{total} = C_{S,O}(e^{-kt}) + C_{R,O}$$

And:

$$C_{S,O} = C_O - C_{R,O}$$

Then:

$$C_{total} = C_{R,O} + (C_O - C_{R,O})e^{-kt}$$

Figure 2. Two-Population Model Kinetics

Hiatt (1964) suggested that survival curves of a two-component character for thermal inactivation are often seen in RNA viruses. A number of factors influence inactivation kinetics (two-population or first order) of viruses, such as a virus's type, structure and composition. For example, enveloped viruses are typically labile to disinfectants, capsid structures or proteins may change in conformation, glycoproteins on the outer surface may be labile to disinfectants, and the nucleic acid type (DNA, RNA, and number of strands) may factor into the inactivation rate of viruses. As stated on earlier, the physical state of the virus also may influence virus inactivation kinetics. Specifically, viruses can adsorb to larger particles; viruses can aggregate together; small particles can absorb to the surface of the

virus; and the virus can be embedded within other material (a membrane or other particle). Protection is enhanced with decreasing size of the microorganism and increasing particle availability. Therefore viruses are more protected than larger bacteria.

The multihit curve is marked by the appearance of an initial shoulder in the survival curve and often indicates either a complex reaction or mechanism based upon cumulative damage. In understanding these data it may be important to understand the number of "hits" required for inactivation or the critical target sites requiring a "hit." Additionally, the magnitude or size of this initial shoulder may also be a function of the number of "hits" (magnitude of cumulative effect) required before the next component of the kinetic reaction with the faster slope is displayed. It should be noted that this multihit curve is also likely to occur as a result of virus aggregation (Hiatt 1964; Sobsey 2003).

Bacteriophages as Indicators of Treatment of Class A Biosolids

Introduction

In 1915 the first phage, *Micrococcus* phage, was discovered by F.W. Twort. Two years later, d'Herelle discovered the *Shigella* phage. Bacteriophages were initially studied in hopes of providing therapy treatment for bacterial diseases because of their ability to kill bacteria (Grabow 2001). It was not until 1948 (Guelin) that the bacteriophages were first advocated as an indicator of fecal contamination (Adams 1959 and Armon and Kott 1996). In the 1950s, Romanian researchers showed *S. typhi* and *E. coli* bacteriophage were correlated with environmental pollution in groundwater (Armon and Kott 1996). Since then, the idea of using bacteriophages as indicators has been explored by many scientists. Male-

specific coliphages are currently being explored as indicators of pathogen removal in thermophilic anaerobic digestion, a process that is being promoted to produce Class A biosolids (Aitken et al. submitted).

Bacteriophages (or phages) are viruses that infect bacteria. Bacteriophages that infect *E. coli* and other coliforms, as well as possibly other members of the Enterobacteriaceae, are referred to as coliphages. Viruses are intracellular obligate parasites and therefore need a host, in this case bacteria, to grow and replicate. All phages have a nucleic acid genome, either DNA or RNA and either single- or double-stranded, surrounded by a protein coat made of multiple copies of one or several distinct proteins called protomers (Grabow et al., 2001; Sobsey et al. 1995). Some phages have a polyhedral head and a special tail structure protruding from the capsid for attachment to and initial infection of the host cell. Based on their mode of interaction with the surface structure of the host bacterium, it is thought that phages can be classified into three groups: those that recognize the bacterium's polysaccharide capsule (capsule phages); those that recognize the bacterium's cell wall (somatic phages); and those that recognize the bacterium's appendages, such as the flagella or pili (male-specific phages) (Furuse 1987).

Additional differences between the bacteriophage families are based on the complexity and contractibility of the tail, if there is one, and its dimension relative to the capsid as well as other morphological, biochemical and molecular properties. There are six taxonomic groups of coliphages. The somatic families include *Myoviridae, Styloviridae* and *Podoviridae* and *Microviridae*. The first three are the tailed bacteriophages consisting of a capsid of cubic symmetry containing ds-DNA and a helical tail. *Microviridae* has cubic symmetry and ss-DNA. The male-specific phages are composed of the Leviviridae family,

which are described by their cubic symmetry and ss-RNA, and the Inoviridae-family, a long (up to 1900 nm) filamentous bacteriophage with more or less flexible rods and ss-DNA. The F+ RNA coliphages can further be subdivided into two genera the leviviruses and alloleviviruses and into four antigenic or genetic groups which are thought to vary in their origins (human or animal) and resistance to environmental factors. The F+ DNA coliphages have received less attention as indicators of enteric viruses because they are sometimes less plentiful than F+ RNA coliphages. They also do not resemble human enteric viruses morphologically, and their ecology is poorly understood (Leclerc 2000).

Indicator Criteria

The current regulations to meet Class A sludge only consider enteric viruses, helminthes, and *Salmonella* (or coliforms). Furthermore, the regulations do not necessarily address all human enteric viruses because the performance criteria of treatment processes are based primarily on the responses of polioviruses and not other viruses. In addition, sludge contains other bacteria pathogens besides Salmonella and it is not clear that Salmonella are representative of all of them. Ascaris ova are the helminths targeted by the current regulations, and as for bacteria and viruses, it is not adequately established that they are a worst case and representative of all parasites.

Below are the criteria for selection of microorganisms for analysis in biosolids or wastewater and the criteria for an ideal indicator.

Criteria for selection of microbes in biosolids or wastewater:

1. Reliable viability assay.
2. Water-related disease causing agents. All selected pathogens must be found in wastewater and should be capable of transmission via exposure (airborne, waterborne, and contact) to biosolids.
3. Extent of existing data on probability of surviving biosolids treatment. The chosen pathogen should have greatest probability of surviving biosolids treatment processes (heat resistant).
4. Extent of survival in the environment. The longer a pathogen survives in the environment, the greater chance of its transmission to a susceptible host.

(Modified from Gerba from NAS 2002.)

Criteria for an ideal indicator:

1. Should be present when pathogens are present and absent when pathogens are absent.
2. Persistence and growth characteristics of the indicator should be similar to pathogens.
3. Indicator should not multiply in the environment.
4. Ratio between indicator and pathogens should be constant.
5. Indicator should be present in greater concentrations than pathogens in contaminated waters.
6. Indicator should be resistant or more resistant to adverse environmental factors, disinfection and other treatment processes as pathogens.
7. Indicator should be non-pathogenic and easy to quantify.
8. Tests for the indicator should be easy and applicable to all types of water.
9. Indicator levels should be associated with the levels of infection or disease risks from pathogens.

*Modified from Goyal (1983) from (Sobsey et al. 1995).

Figure 3. Criteria for Selection of Microbes in Biosolids or Wastewater and Criteria for an Ideal Indicator

It should be noted that there is confusion between the definition of an indicator organism and an index organism in much of the literature. For consistency purposes, the term "indicator" was used when referring to the usefulness of an organism in modeling pathogen reductions in a water treatment process, such as thermophilic anaerobic digestion. In this paper, it does not imply or indicate fecal or sewage contamination.

Armon and Kott (1996) summarize the differences between an indicator and index organism. They state that an indicator organism is a model that portrays similar behavioral characteristics to pathogens, illustrates the same or greater resistance to environmental stresses, and can be used to evaluate the efficiency of treatments. An index organism is described as an organism related to pathogenic microorganisms by a health risk or occurrence of illness. This relationship can be direct or indirect, such as an index of fecal pollution or sewage contamination. Based on these definitions we will refer to bacteriophages as an "indicator" of pathogen reductions in thermophilic anaerobic digestion.

Since the beginning of the century, various microorganisms such as *E. coli*, *Steptococcus faecalis*, and *Clostridium perfringens*, have been used as indicators of water pollution. As seen above, coliforms and fecal coliforms are still accepted as an indicator of pathogen reductions in PFRP for Class A biosolids. However, it has been suggested that bacteria (i.e. coliforms) differ in their resistance to environmental conditions and sewage or water treatment processes, compared to viruses (Armon and Kott 1996; Leclerc et al. 2000; NAS 1996).

First, the morphology of bacteria is different from viruses. For example, coliforms are cellular and viruses are acellular. The acellular nature of enteric viruses makes them less subject to environmental stress than coliforms. Thus enteric viruses tend to survive longer in the environment than do bacteria (Funderburg and Sorber 1985). Additionally, viruses are smaller than bacteria (Leclerc et al. 2000) and carry a charge on their surface that will allow them to readily adsorb to solids under the right ionic conditions. This adsorption may also protect viruses from inactivation better than the unabsorbed bacteria. Thus the ultimate distribution of viruses and bacteria in water or wastewater may be different (Funderburg and Sorber 1985). However, *E .coli* and fecal coliforms continue to be employed because they can be monitored frequently, inexpensively, and with simple, specific tests. These bacteria may be a more appropriate indicator of enteric bacteria such as *Campylobacter, Salmonella, Shigella, Yersinia*, and *Vibrio* than enteric viruses, such as those belonging to the *Caliciviridae* or *Adenoviridae* families (Sobsey et al. 1995).

It has been demonstrated that F+ RNA coliphages fit many of the criteria for viral indicators. These viruses attach to bacteria through receptor sites on hair-like surface appendages of the bacteria called pili (Leclerc et al. 2000). The pili are produced by the

bacteria only when they grow at higher temperatures (i.e. in warm-blooded hosts). Presumably bacteria with pili originate only in the intestines of warm blooded animals (Cole et al. 2003), and therefore their presence in the environment is taken as evidence of fecal contamination (Sobsey et al. 1995).

F+ RNA coliphages superficially resemble Enteroviruses (Family Picornaviridae) such as polioviruses, coxsackieviruses and echoviruses because they consist of ss-RNA surrounded by a protein coat, icosahedral shape, and small size (~25 nm diameter). It has been reported that the two are almost indistinguishable under the electron microscope (Grabow 2001). F+ RNA coliphages are thus expected to exhibit similar persistence and survivability in the environment and through treatment processes as enteric viruses such as the enteroviruses, noroviruses, astroviruses and hepatitis A and E viruses (Havelaar 1993). It has also been demonstrated that these morphological similarities may potentially be useful for indicating enteric virus removal in activated sludge treatment and other waste treatment processes (Funderburg and Sorber 1985).

Additionally, F+ RNA coliphages can be enumerated by simple, inexpensive, precise methods and results may be available within 6-8 hours (Debartolomeis and Cabelli 1991; Gerba 1987; Havelaar 1987; Funderburg and Sorber 1983; Leclerc 2000). They are consistently abundant in sewage (mean $>10^3$/ml; range: 10^2-10^5/ml) and both treated and untreated sewage effluents, and sewage-polluted waters (Debartolomeis and Cabelli 1991; Leclerc et al. 2000; Havelaar et al 1990). They are also consistently more resistant to inactivation by environmental factors and inactivation processes than the traditional fecal bacteria (*E. coli*, fecal streptococci, fecal coliforms) (Havelaar 1993) and their resistance appears to parallel or slightly exceed that of culturable enteric viruses. Additionally, like

enteric viruses they fail to replicate in the environment and almost exclusively originate from the feces of humans and other warm-blooded animas (Grabow 2001; Woody and Cliver 1995; Sobsey et al. 1995).

Phages can be easily and cheaply detected by either the single or double agar layer plaque methods. These methods are similar to the simple pour plate methods for bacteria except for the addition of host bacteria to support virus replication that leads to the development of discrete plaques in analogy to bacterial colonies. In samples where their numbers are too low to be detected by these plaque methods, phages can be enriched to provide enough viruses for direct plating assays, for electron microscopy examinations, immunoassays or molecular detection. These methods allow phages to be quantified relatively quickly (< 24 hours), compared to methods used to evaluate many individual human enteric viruses. For example, analysis of Hepatitis A virus infectivity can take over a week for cell culture adapted strains and several weeks for field isolates.

Methods, such as hydroextraction, centrifugation, ultrafiltration, adsorption to precipitates (magnetite-organic flocculation) and procedures using microporous filters, have been developed to concentrate human enteric viruses as well as bacteriophages from different types of water (IAWPRC 1991; Armon and Kott 1996). Most recently, molecular techniques for phage detection have been gaining use, but many of the methods are still under development and being evaluated.

While literature states F+ RNA and F+ DNA coliphages are rarely found in human feces (Leclerc et al. 2000; Havelaar and Hogeboom 1984; IAWPRC 1991), they are consistently found in high concentrations in raw sewage. This discrepancy will be discussed later below. While both F+ RNA and F+ DNA phages have been found in sewage, the

sanitary significance of the F+ DNA phages has not been as clearly determined as that for the F+ RNA coliphages (Leclerc et al. 2000). In part, this is because the F+ DNA phages have received less attention than F+ RNA phages, probably due to their different morphologically from enteric viruses, and their ecology is still unclear (Leclerc et al. 2000).

The reasons for the difference in isolation between humans and the higher numbers found in sewage have not been resolved (Leclerc et al. 2000). As previously noted, studies have detected F+RNA coliphages 10^2-10^5 pfu/ml in raw swage (Furuse 1987; Havelaar 1986, 1990; IAWPRC 1991; Debartolomeis and Cabelli 1991). The discrepancy between studies on F+ RNA coliphage presence in feces versus sewage may be attributed to several factors. One is the likelihood that the F+ RNA coliphages shed by the rare (1-5% prevalence) infected or colonized individuals undergo a cycle of replication in available host bacteria in fresh sewage, thereby creating a relatively high and constant presence in raw sewage. In addition, the incidence and survival of phages in feces and sewage is subject to many variables and there are different techniques used by different laboratories to enumerate the phages (Grabow 2001).

It also has been postulated that the conditions in sewage may permit efficient phage multiplication. However infection by phages of F+ *E. coli* host cells is initiated by attachment to the F pili, which are synthesized only under certain conditions of growth of the host. F pili are not produced below 25 °C (Novotny and Lavin 1971) and are maximally produced at 37-42 °C (Havelaar 1986; Novotny and Lavin 1971). Woody and Cliver (1995) demonstrated that below 25 °C F-pilus synthesis ceases and F+ RNA coliphage Qβ did not replicate below this temperature in batch cultures. F-pili are synthesized by exponentially growing host cells, with maximum piliation occurring in late logarithmic growth stages. Pili

are lost as cells progress into stationary phase. However, conditions, even in sewage, rarely if ever meet requirements for the production of fertility fimbriae, especially in cooler climates. Thus the dependence of F+ RNA coliphages on temperature and temporally regulated F-pili production and presence restricts the replication of these phages (Woody and Cliver 1995; Grabow et al. 1995). This is an important limiting factor for their replication and implies that for all practical purposes continued or sustained replication of male-specific phages in environmental waters and sewage is unlikely (Grabow 2001). However, F+ cells grown at temperatures above 30 °C may remain piliated when released in the environment. Havelaar and Pot-Hogeboom (1988) found that F+ RNA coliphage group II (GA) could multiply at 20 °C if the host cells were grown at a higher temperature of 37 °C and therefore had pili. These results support the suggestion of Havelaar et al (1986) that multiplication may occur in raw sewage where host cells originating in the human intestines were added to the system. Furthermore, it should be noted that phages require a host bacteria concentration of 10^4 ml^{-1} and it is not likely that multiplication will take place at environmental sites other than the sewerage system (IAWPRC 1991).

It is additionally important that a successful candidate indicator be present when the pathogen is present. In a survey of a range of wastewaters and raw water sources, F+ RNA phages have been found to outnumber cytopathogenic enteric viruses by a factor of about 100 (Grabow 2001). In another study, Simkova and Cervenka (1981) compared coliphages to enteric viruses in four types of water: sewage, irrigation channels, river waters and wells. They found similar concentrations of coliphages and enteric viruses and noted that the seasonal variation for coliphages and enteric viruses followed the same pattern (Sobsey et al. 1995). Similar findings have been recorded by Grabow (1990) on phages and viruses in

advanced treatment trains for the direct reclamation of drinking water from waste water. This implies that the absence of F+ RNA phages from raw and treated water supplies offers a meaningful indication of the absence of human enteric viruses (IAWPRC 1991; Grabow 2001).

The indicator function of bacteriophages requires that their resistance to treatment processes be similar to or slightly greater than that of the relevant pathogens. Many experiments confirmed that the resistance of F+ RNA coliphages to unfavorable conditions and disinfection processes resembles or exceeds that of most human enteric viruses (Grabow 1990; IAWPRC, 1991; Havelaar et al 1993). Nassar and Oman (1999) demonstrated that *E. coli* have greater inactivation when exposed to a variety of environmental factors than that of male-specific coliphages. Additionally, Stetler (1984) illustrated that enteric virus isolations from a drinking water treatment plant were better correlated with coliphages than with total coliforms, fecal coliforms, fecal streptococci, or standard plate count organisms from the source, post-sedimentation, and post-sand filtration processes.

Heat Inactivation of Bacteriophages

In order to use F+ RNA coliphages is an indicator of pathogen reductions in thermophilic anaerobic digestion, it is important to assess their thermal inactivation properties. Viruses are composed of nucleic acid cores embedded within protein capsids, with or without external envelopes. The nucleic acid is the genetic material responsible for the replication of virus within the host cells. Envelopes and capsids are protective structures which also play roles in the initiation of virus infection by acting as receptors of virus binding sites. Infectivity may be lost either because of an alteration in the viral genome

leading to failure to produce normal viral progeny or because of damage to the outer layers of the virons, resulting in failure to attach to cells, to penetrate, or to initiate replication and maturation. (Luria 1953).

Bleichrodt et al. (1968) concluded higher temperatures may cause damage to viral capsids by either denaturation, or protein destruction and is dependent on the structure of the adsorption sites (Nasser and Oman 1999), or microbial degradation which results in the liberation of the viral RNA. Limsawat and Ohgaki (1997) demonstrated that the free viral RNA in wastewater could disappear in a few minutes after being released from the Qβ coliphage. However, the persistence of released viral nucleic acid in various environmental media is poorly understood and some evidence indicates long-term persistence for as long as months.

Temperature influences microbial disinfection kinetics, and in general increased temperature produces increased rates of inactivation (Sobsey 1989). Studies have shown that inactivation of bacteriophages is temperature-dependent (Yates et al 1985; Armon and Kott 1996; Niemi 1976; Mignotte-Cadiergues et al. 2002). Conditions that denature proteins or nucleic acids and thereby inactivate phages, such as heat, tend to be phage-specific, and sometimes help in the classification of phages (Adams 1959). Like, the majority of non-spore-forming bacteria, phages are inactivated almost instantaneously at 100 ^{o}C. Between 60 ^{o}C and 85 ^{o}C, inactivation rates can be determined conveniently under laboratory conditions and used to characterize phages. Below 65 ^{o}C, some phages are inactivated fairly rapidly (within seconds to minutes) but others hardly at all. D'Heurell (1926) noted that several phages were inactivated by heating at 75 ^{o}C for 30 minutes whereas some survived after heating at 70 ^{o}C (Adams 1959).

Observations of this type led to the notion of a qualitatively defined inactivation temperature, characteristic for each phage, analogous to thermal death point used to report the results of similar tests with bacteria (Adams 1959). Quantitative study of the effects of heating showed that phages are inactivated in accordance with first-order kinetics (Adams 1959).

The medium in which phages are heated has a great influence on the rate of inactivation. Inactivation is most rapid in pure water; the addition of salts, especially calcium or magnesium or proteins reduces the rate of inactivation considerably. For example coliphages may be inactivated ten times faster in saline than in broth (Adams 1959). Additionally, Nasser and Oman (1999) suggest wastewater may provide general protection against the inactivation of microorganisms or enhance virus persistence.

While heat does inactivate coliphages, it has been shown they are more resistant to thermal inactivation than any bacteria, except that of bacterial spores, such as those of sulfite-reducing clostridia.

Moce-Llivina et al. (2003) studied the inactivation of naturally occurring bacterial indicators and bacteriophages by thermal treatment of dewatered sludge and raw sewage. The sludge was heated at 80 °C and the sewage was heated at 60 °C. In both cases phages were significantly more resistant to thermal inactivation than bacterial indicators, with the exception of *Clostridium perfringens* spores. They additionally looked at the thermal treatment of various phages after they were added to the sewage. The results revealed variability between the phages studied and that the *B. fragilis* and somatic phages were more resistant than MS2 (an F+RNA phage) after 30 minutes at 60 °C (Moce-Llivina et al. 2003).

Finally, it is worth noting that other factors, besides the heat, are likely to be contributing to the inactivation of viruses in the thermophilic anaerobic digester. These include pH, concentrations of protonated (non-ionized) organic acids, and free NH_3 (non-ionized ammonia). The effects of ammonia have been discussed to some extent in the literature.

Ammonia is a viricide and may be effective at inactivating viruses in wastewater treatment plants; evidence for the antiviral effect of ammonia is especially good for polioviruses. The bacteriophage f2 is inactivated by ammonia but the rate of inactivation is 4.5 times slower that of poliovirus type 1 strain CHAT (Cramer et al. 1983). Furthermore, the inactivation of both f2 and poliovirus by ammonia is strongly influenced by temperature, especially in ranges found in a thermophilic anaerobic digestion (Burge et al. 1983). Schaper et al. (2003) compared the inactivation rates of the F+RNA coliphage groups to ammonia at pH=10 and found the group I coliphages inactivated to a lesser extent (about 1 $\log_{10}$ less) than groups II, III, and IV.

Shortcomings of Bacteriophages as Indicators

There are also a number of shortcomings in the use of bacteriophages as indicators. While there are many studies on phages and their survival rates, there is also considerable variability in data. This is partially due to the inconsistencies in techniques used for the recovery and assay of phages from water environments; the variations of host bacterium used to analyze the groups of phages; and the different detection and enumeration methods used among various laboratories (Grabow 2001 and Duran et al 2002).

As mentioned earlier, there are many and varied agents which cause viral gastroenteritis. Unfortunately, data on the transport and die-off of many of these is scarce.

Some can be grown in culture and some cannot (Scott et al. 2002). Until there are data on these enteric viruses, it will be difficult to accurately compare them to bacteriophages.

It has been well-established that there are some differences between certain bacteriophages and enteric viruses. Electrostatic charges, for example, on phages may differ from those on enteric viruses, which affect important properties such as adsorption to solid surfaces. This has implications for features like behavior in the environment and the efficiency of recovery by techniques based on adsorption-elution principles (Grabow 2001).

Literature Review Conclusions

An indicator is needed to monitor the efficiency of pathogen reduction in PFRP to meet Class A sludge standards. F+ RNA coliphages may be particularly useful as virus indicators for thermophilic anaerobic digestion processes. These organisms are morphologically similar to enteric viruses, are present in sewage, are similar or more resistant to inactivation processes than enteric viruses, and can be monitored in an easy, fast, and cost-effective manner. Because of considerable gaps and inconsistencies in the data describing the relationships between coliphage and human enteric viruses' responses to thermophilic anaerobic digestion processes, additional studies are needed to develop such data.

III MATERIALS AND METHODS

Experimental Design

Thermophilic Anaerobic Digester (TAD)

A small (20 L) continuous-flow thermophilic anaerobic digester was operated to simulate the main reactor in the Columbus Biosolids Flow-Through Thermophilic Treatment (CBFT3) process. The plug flow reactor, the second step in the CBFT3 process, was simulated by treating effluent from the continuous reactor in a small batch reactor. Two batch treatment tests were conducted for each operating condition evaluated. The combination of continuous plus batch thermophilic treatment was evaluated at 55 °C, 53 °C, and 51 °C with sludge from the South Columbus (GA) Water Resources Facility (SCWRF). The concept was also evaluated at 53 °C with fermented primary sludge from the Orange Water and Sewer Authority (OWASA) wastewater treatment plant in Chapel Hill, NC and with waste activated sludge from the Western Lake Superior Sanitary District (WLSSD) wastewater treatment plant in Duluth, MN. General characteristics of the three sludge sources are summarized in Table 3.

The hydraulic retention time (HRT) and the solids residence time are the same in a mixed, continuous flow reactor without recycle, and are defined as the reactor volume divided by the average flow rate. The target residence time at the beginning of the study (operation with SCWRF sludge at 55 °C) was four days, but was increased to between 5.3 and 6 days for all other operating conditions. These residence times are shorter than those

normally used for single-stage anaerobic digestion, including thermophilic anaerobic digestion. Accordingly, the short residence times used were meant to provide conservative data on pathogen inactivation in single-stage continuous-flow digesters.

Table 3. Characteristics of Sludge Sources Used in the CBFT[3] Project

Source	Sludge Type	Average Plant Wastewater Flow (mad)	Approximate Contribution from Industries (%)	Primary Industrial Activity
SCWRF	primary + waste activated [a]	28	20	textile, bakery
OWASA	fermented primary	9	< 2	university, hospital
WLSSD	waste activated [b]	40	50	pulp and paper

[a] Approximately 55% primary, 45% waste activated (v:v).
[b] From a pure-oxygen plant with no primary treatment.

At least two samples of feed sludge and effluent biosolids were obtained for each operating condition for analysis of *Ascaris suum*, poliovirus, *Salmonella* spp., fecal coliforms, *Clostridium perfringens* spores, somatic coliphages, and male-specific coliphages. *Salmonella* spp. and fecal coliforms are approved alternative indicators for monitoring Class A biosolids (US EPA, 1999). *C. perfringens* spores and the coliphages were included as other potential indicators of pathogen inactivation in thermal treatment processes. The inactivation of coliphages may be indicative of human enteric virus inactivation.

Two batch treatment tests were conducted on effluent from the continuous reactor at each operating condition. During a batch treatment test, samples were withdrawn at selected time intervals and analyzed for the same microbial constituents as were analyzed in the samples from the continuous reactor. The number of samples in the batch tests was selected on the basis of minimum sample size required for the analyses. The sampling intervals were selected to try to capture reductions in target species to below detection limits in at least one

sample in the absence of prior information on concentrations in the effluent or on inactivation rates.

Separate tests were conducted to measure the rate of inactivation of *Ascaris*, poliovirus and coliphages in treated biosolids from the continuous-flow digester. In these tests, biosolids were transferred to the batch reactor and then spiked with the organisms of interest. Samples were collected at selected time intervals as described above. The design and operation of the continuous reactor, including the temperature control and monitoring system is described elsewhere (Aitken et al., submitted).

Male-Specific Bacteriophage Characterization

After the untreated and treated sludge samples were processed and the bacteriophages were enumerated using Most Probable Number (MPN), coliphage isolates were collected for characterization. The following methods were used: RNAse suppression of F+ RNA coliphages, F+ RNA serotyping with antisera, Polymerase Chain Reaction (PCR), Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR), and Reverse Line-Blot Hybridization (RLB-Hybridization).

Lab-Scale Heat Inactivation Experiments at 53 °C

Once the characterization was accomplished, a sample of each coliphage isolate type was chosen for heat inactivation experiments. F+ RNA (groups I and III) and F+ DNA coliphages were suspended in bacteriological broth and replicate volumes placed in microcentrifuge tubes were exposed to a thermoregulated heat block for 5 minutes and 60 minutes at 53 °C. A heat inactivation rate was then estimated for each isolate.

Thermophilic Anaerobic Digester (TAD)

Batch Tests

Batch treatment of effluent from the continuous digester was evaluated by transferring approximately 4.5 L to the batch reactor. Transfers were performed anaerobically by purging all transfer tubing and the batch tank with argon, closing all ports to the continuous digester, pressurizing it with argon, then opening a transfer line between the continuous digester and the batch tank. The effluent tubing for the batch reactor was kept open and the reactor was purged with argon during the transfer. Samples were collected at the time of filling (time zero) and at subsequent time points that depended on the temperature being evaluated.

Sample Collection

Feed samples were collected by closing the feed tubing from the continuous reactor inlet and pumping the sludge into a sterile 1-L polyethylene bottles and placed in a cold room (approximately 5 °C) until transported to the microbiology laboratory. Feed samples were prepared for microbial analyses within 48 hours of collection.

Most samples of effluent were collected on the same day as the batch tests. Only one sample of effluent was not collected on the day of a batch test; in that case the sample was obtained by pressurizing the continuous reactor and displacing biosolids through the effluent port. Biosolids samples from each batch test and each inactivation rate test were transported in a cooler containing refrigerant packs to the microbiology laboratory at the completion of the test. All shipments were accompanied by chain-of-custody forms that were initiated by personnel responsible for sampling and transport and were completed by personnel in the

microbiology laboratory. All biosolids samples except feed samples (see previous paragraph) were prepared for microbial analysis within 30 hours of collection.

Preparation of coliphages for spiking

Coliphages were spiked into biosolids samples for the inactivation rate experiments. The coliphages were concentrated from raw wastewater collected at the wastewater treatment plant in Beaufort, NC. Dr. Mina Shehee kindly concentrated the coliphages by ultrafiltration in a 25 ml Amicon Ultra-15 Centrifugal Filter Device with a 10,000 nominal molecular weight cutoff (Millipore, Billerica, MA). The filter devices were centrifuged at 4,000 x g for 20 minutes at 4 °C.

Inactivation rate experiments

To initiate an inactivation experiment, approximately 4.3 to 4.5 L of biosolids from the continuous digester was transferred anaerobically to the batch reactor. The temperature was allowed to stabilize to the desired temperature before spiking the reactor with the pathogen surrogates. Except for the first experiment at 55 °C, the mixing speed in the reactor was increased before spiking to ensure rapid mixing of spiked organisms. The mixing speed was turned down before the first sample was collected. Poor mixing in the first experiment at 55 °C may have compromised the data from samples collected over the first 15 minutes, so data from that experiment are interpreted and discussed only qualitatively.

A 100 ml aliquot of spiking material was prepared by mixing concentrated stocks of Ascaris, poliovirus, and coliphages into untreated sludge (feed sludge for the continuous digester). The approximate volumes were 60 ml of sludge and 40 ml (combined) of the pathogen and coliphage stocks. Target amounts of Ascaris and poliovirus added in the spike

were 4 x 10^5 eggs and 10^6 pfu, respectively, which corresponded to initial concentrations in the reactor of 3.6 and 4.0 $\log_{10}$/g TS, respectively.

The spiking material was mixed in a beaker, covered with Parafilm, and stored cold overnight. It was removed from the refrigerator two to three hours before initiating the batch test and allowed to approach room temperature, then was warmed slowly with slow mixing (just below the speed required to create a vortex at the liquid surface on a hot-plate). For the first test at 55 °C, the spiking material was warmed to 40 °C and for all subsequent tests it was warmed to approximately 35 °C. The spiking material was removed from the hot plate immediately after reaching the target temperature, then poured into the batch reactor through a wide port. A stream of argon was delivered to the batch reactor while the port was open. The port was then closed and the reactor contents allowed to mix for 90 seconds before collecting the first sample. This sample is referred to as the "time zero" sample. Subsequent samples were collected at pre-selected time intervals. All samples were cooled and processed as described above, except the maximum aliquot volume was approximately 300 ml per sample bottle.

Samples representing successive time points were analyzed for Ascaris until no viable organisms were detected. Poliovirus was measured at most of the time points, and all samples were analyzed for coliphages and total solids. In some cases the last samples collected had significantly higher solids content than the earlier samples, suggesting that the small volume remaining in the batch reactor was not mixed well. By this time the samples were only analyzed for coliphages and we do not believe that a lack of mixing influenced the results significantly.

Male-Specific Coliphage Analysis from Municipal Sludge

Hosts and Media

Male-specific coliphages were quantified in accordance with the procedure outlined in EPA 821-R-01-030, Method 1601: Male Specific (F+) and Somatic Coliphage in Water by Two-Step Enrichment Procedure (US EPA 2001). A few modifications were made to these methods. First, a instead of testing for presence/absence, Most Probable Number (MPN), was used to quantitatively evaluate the results. Further, Method 1601 calls for the use of one liter sample volumes. In this assay we used variable volumes of sludge and biosolids, depending on the expected range of coliphage concentrations present. In cases of higher coliphage concentrations, smaller volumes (100 ml) were sufficient.

Briefly, bacterial hosts (American Type Culture Collection, Manassas, VA) for the male-specific phages (*Escherichia coli* F_{amp}; ATCC No. 700891) were inoculated in tryptic soy broth (TSB) medium (Difco) supplemented with antibiotics relevant to host selection and incubated at 37 °C overnight (12-18 hours) on a rotary shaker (100 to 150 rpm). The TSB medium consisted of 3g of tryptic soy broth per 100 ml reagent water. The antibiotic supplement stock for the male-specific host consisted of 0.15 g ampicillin (Sigma) and 0.15 g streptomycin (Sigma) in 100 ml of reagent water that was filter-sterilized using a syringe and a 0.2 μm HT Tiffryn membrane (Gleman) and stored in the -20 °C walk-in freezer. A 10-ml volume of the stock was diluted into 1 L of TSB to prepare the host medium. After overnight incubation 1 ml of each host culture was transferred aseptically to 50 ml of fresh medium and incubated at 37 °C for four hours on a rotary shaker (100 to 150 rpm). These cultures served as log-phase cultures for inoculation of enrichment tubes as described below. *E. coli* F_{amp} has three antibiotic resistance markers, ampicillin on the F_{amp} plasmid, which codes for pilus

production, streptomycin, and naladixic acid on the chromosome. This host bacterium is thought to have high plaquing efficiency in stock suspensions and sewage effluents (Debartolomeis and Cabelli 1991).

Inoculation

Samples were initially mixed in a Waring® blender on high for one minute and then stored at 4 °C until processed. Decimal dilutions of each sample were prepared in PBS in five-tube MPN format. At least three dilutions were made for each sample. A 1 ml aliquot of each sample or dilution was then added to 9 ml of enrichment broth media, which consisted of TSB supplemented with antibiotics as described above in a sterilized 16 x 125 mm glass tube. Each enrichment tube was supplemented with 100 μl of the log phase F_{amp} host culture, and then was incubated at 37 °C for 18 to 24 hours. Negative controls (host with no sample added to TSB and antibiotic) and positive controls (host with appropriate control phages added to TSB and antibiotic) were included for each male-specific coliphage assay. The male-specific positive control used was group I (MS2).

Phage extraction

After incubation, 0.6 ml from each tube was transferred into a 1.5 ml-sterile Eppendorf® tube. The phages were extracted by adding 0.3 ml of chloroform, vortexing, and centrifuging at 3,000 rpm for 1 minute. Aliquots of 10 μl of the aqueous layers were spotted onto plates containing tryptic soy agar supplemented with the appropriate antibiotic and a lawn of the bacterial F_{amp} host. A given plate contained spots for all five tubes at each dilution for a given sample (e.g., 20 spots were made if four dilutions were made for a given sample). Plates were marked and aligned with a grid to identify the locations of specific

spots. The spotted inocula were allowed to absorb into the medium for 30 to 60 minutes, then the plates were inverted and incubated at 37 °C for 18 to 24 hours. After incubation, the spot plates were examined for zones of lysis (clearing). Each zone of lysis was considered to represent a positive enrichment tube.

The medium for the spot plates consisted of 3 g TSB and 0.75 g agar per 100 ml reagent water. After autoclaving and cooling to 45 °C, 1 ml of stock antibiotic solution and 2 ml of the corresponding log phase F_{amp} bacterial host culture were add per 100 ml. The medium was then distributed at 30 ml per plate and allowed to solidify. This is the method used to make tryptic soy agar (TSA) plates during the entirety of the project.

Bacteriophage Enumeration Technique: MPN method

The Most Probable Number (MPN) procedure is a statistical method based on diluting organisms to disappearance and evaluating whether a positive sign of the organism's presence appears in replicate tubes for a give dilution. The multiple tube inoculation technique may be used to obtain statistically valid MPN estimates of coliphage density.

In this examination of biosolids, a series of five tubes were inoculated with decimal quantities of biosolids based on the probable bacteriophage density. The results from three of these dilutions were then used in computing the MPN. To select the three dilutions to be used in determining the MPN index, the highest dilutions that gave positive results in all five portions tested and the two next succeeding higher dilutions were selected. If fewer than 5 tubes were positive in any dilution, then the dilution with the highest number of positive tubes plus the next two dilutions was selected for determination of the MPN.

Statistical Analysis of Data for (TAD) Continuous and Batch Samples

MPNs, means, and standard deviations of data sets were calculated with Excel with the help of Dr. Mina Shehee. For the microbial constituents, means and standard deviations were determined from the $\log_{10}$ values (geometric means and standard deviations). Standard deviations of calculated values were determined with propagation of error formulas from the means and standard deviations of the individual values (Young 1962). Comparisons between means were made with t-tests for independent samples using TK Solver 4.0 (Universal Technical Systems, Rockford, IL). Differences between means are considered significant if $p \leq 0.05$. Linear regressions were performed either with Grapher (Golden Software Inc., Golden, CO) or with Excel. Non-linear regression was performed with ProStat (Poly Software International, Pearl River, NY).

Male-Specific Coliphage Characterization

Isolation

Representative plaques from *E. coli* F_{amp} streptomycin-ampicillin tryptic soy agar (TSA) plates were picked from municipal sewage sludge samples taken from the thermophilic anaerobic digester. A 500 µl portion of TSB was pipetted into an autoclaved 1.5 ml Eppendorf® tube. A filtered pipet tip was then touched into the plaque and bacteriophage was drawn up using a pipettor Pipetman (Gilson, Campbell, CA). The pipet tip was then dipped into the labeled tube and the virus was released into the TSB using three pumping motions. The tube was then vortexed.

The 500 µl portion was separated equally into four different autoclaved 1.5 ml Eppendorf® tubes (125 µl was placed in each tube). Two of the tubes were immediately stored at -20 °C as archive samples. One drop of log phase *E. coli* F_{amp} host was added to the remaining two tubes to enrich the phage. These two tubes were then placed in incubators

overnight at 37 °C. The following morning they were transferred to the -20 °C freezer until use. This process was repeated for every phage isolate.

RNAse Test

The nucleic acid of male-specific isolates, those infecting F+ bacteria, was differentiated by testing for growth in the presence of RNAse, an enzyme which inhibits the activity of RNA. One tube containing each isolate in TSB only was taken out of the freezer and allowed to thaw on ice. Employing the same technique for preparing spot plates, two bottles of TSA were made with the appropriate antibiotics and host. However to one of the bottles, 1 ml of RNAse (Type 1-A, Sigma-Aldrich; St. Louis, MO) solution, made up at 10 mg/ml, was also added per 100 ml of TSA. The media was again distributed at 30 ml per plate. There were an equal number of regular TSA plates and TSA plates with RNAse added.

After the plates dried, 10 μl of each unenriched male-specific coliphage isolate was spotted on each of the solidified agar medium-host bacteria plates. The plates were gridded and numbered for isolate identification purposes. The 10 μl phage drops were allowed to dry, inverted, and incubated overnight at 37 °C. The next morning, plates were examined for lysis zone formation. Lysis zone formation on the *E. coli* F_{amp} and the *E. coli* F_{amp} plus RNAse plates was to indicate F+ DNA coliphages, members of the *Inoviridae* family. F+ DNA coliphages were expected to grow equally well on the plates with and without RNAse. Lysis zone formation of the *E. coli* F_{amp} plate but not the *E. coli* F_{amp} plus RNAse plate was to indicate F+ RNA coliphages, members of the *Leviviridae* family. A positive control F+ RNA (MS2) was also spotted on both of the plates, as were the negative control blanks for quality insurance.

Serological Typing of F+RNA Coliphage Isolates

F+ RNA coliphage isolates were subdivided into one of five groups (4 major groups: I, II, III, and IV) using spot neutralization tests with antisera raised in rabbits against prototypical viruses of each group. The groups were: I (MS2), II (GA), III (Qβ), IV (SP), IVa (FI). The latter two subgroups of IV are closely related bacteriophages. Again, TSA plates were prepared as above, for spotting. Antisera were added to the medium of individual 10-cm diameter Petri plates at concentrations optimal for neutralization of virus infectivity. These concentrations varied between 0.1 ml of a 10^{-1} dilution and 0.5 ml of a 10^{-2} dilution, are specific for the particular antiserum stock and were kindly determined for these stocks at UNC by Douglas Wait. Six plates were prepared, one containing each antiserum and one containing no antiserum. Grids were drawn with a ruler. One tube of each unenriched F+ RNA phage isolate was allowed to thaw on ice. Then, 100-fold serial dilutions were then made of each F+ RNA isolate (10^{-2}, 10^{-4}, and 10^{-6}) in PBS. An 8 μl portion of each dilution of each isolate was pipetted onto corresponding squares of each plate (undiluted, 10^{-2}, 10^{-4}, and 10^{-6}). The plates were allowed to dry for 30-60 minutes and then inverted and incubated overnight at 37 °C. The absence of lysis zone on a lawn was considered complete neutralization of phage infectivity by the antiserum in that plate and positive identification of the phage serotype if the same isolate was not neutralized by any other antisera (lysis present). F+ RNA group I (MS2) was used as a positive control.

Detection of F+ RNA phages by broadly-reactive duplex RT-PCR

When the RNAse test produced ambiguous results, genetic typing of F+ RNA coliphage isolates was performed by hybridization assays after RT-PCR amplification of

viral nucleic acid. Genetic typing uses non-radioactive oligonucleotide probes to subgroup F+ RNA phages isolates into one of the four known groups: I, II, III, IV. These groups are analogous to the groups identified by serological testing.

RT-PCR for leviviruses (group I and II) and alloleviruses (group III and IV) was performed as described (Vine et al. 2004) using the One-Step RT-PCR kit™ (Qiagen) according to the manufacturers' instructions. Briefly, enriched phages were first diluted 1:50 in RNAse/DNAse-free water (Sigma-Aldrich) followed by heat release of viral nucleic acid for 5 minutes at 99 °C, quenching on ice for 2 minutes, and then spun for 5 seconds. For reverse transcription (RT) and amplification, 2.5 µl of the lysate was added to the One-Step RT-PCR mix that was aliquoted (22.5 µl per reaction) in ultra-thin sterilized PCR tubes (Sarstedt). The final volume of 25 µl consisted of 1X buffer, 0.4 mM dNTP mix, 0.6 µM JV41 primer, 0.6 µM JV81 primer and 0.8 µM MJV82 primer, 20 U RNAse inhibitor (Promega, Madison, WI) and 1 µl of Enzyme Mix. Primers MJV82 and JV81 will amplify leviviruses and MJV82 and JV81 will amplify alloleviviruses. MS2 (group I) and Qβ (group III) were included as positive control. RT was performed for 30 minutes at 42 °C, followed by activation of the Taq polymerase for 15 minutes at 95 °C. The samples were then subjected to 40 cycles of 30 seconds at 94 °C, 30 seconds at 45 °C and 30 seconds at 72 °C. A final extension was performed for 10 minutes at 72 °C. The thermocycler program is outlined in Table 4 below.

Table 4. RT-PCR Thermocycler Program

Step (ALL-Levi program)	Temperature	Time
Reverse Transcription	42 °C	30 min
Initial PCR Activation (1 cycle)	95 °C	15 min
PCR (40 cycles)		
Denaturation	94 °C	30 sec
Annealing	45 °C	30 sec
Extension	72 °C	30 sec
Final Extension (1 cycle)	72 °C	10 min
Hold	4 °C	20 min

Success of the RT-PCR reactions and positive identification of isolates as levivirus or allolevivirus was confirmed by electrophoretic analysis on a 2% ethidium bromide-stained agarose gel. Leviviruses (MS2 (group I) and GA (group II)-like viruses) yielded a 266 bp product and allelolevivirus (Qβ (group III) and FI/SP (group IV)) yielded a 229 bp product. Gels were typically electrophoresed at 75 volts for 1 hour. Positive controls and negative control reactions (without template RNA) as well as molecular weight markers, consisting of a 100 bp DNA ladder (Promega® Madison, WI) were included on each gel.

Genetic Detection of F+DNA Coliphage Isolates

When the RNAse test produced ambiguous results for distinguishing F+ DNA and F+ RNA coliphages, PCR-RLB typing of F+DNA coliphage isolates was also performed. A mastermix was prepared on ice using reaction components listed in the Table 5. This mix consisted of PCR buffer pH 9.0 (Promega), 0.2 mM dNTP, 15 pmol of SL-2 primer and 15 pmol of SL-3 primer, 1.25 U of Taq (NEN Biolabs), 1.5 mM of $MgCl_2$. The SL2 and SL3 primers were designed to amplify all F+ DNA viruses (Vine et al. 2004).

Table 5. Master Mix Components for PCR Reactions

Component	Volume/Reaction	Final Concentration
RNAse-free water (QIAGEN)	19.60	
10X PCR buffer pH 9.0 (Promega)	2.50	
PCR nucleotide mix (10 mM)	0.50	0.2 mM
SL-2 primer (50 pmol/μL)	0.20	15 pmol
SL-3 primer (50 pmol/μL)	0.20	15 pmol
Taq (5 U/uL)	0.25	1.25 U
MgCl2 (50 mM)	0.75	1.5 mM
Total volume / reaction	24	

After the phage DNA was heat released one μl of each phage isolate was added to the master mix. Positive (M13) and negative (water) controls were included in each experiment. The PCR tubes were then transferred from ice to a preheated (>80 °C) thermocycler. The thermal cycle program is outlined in the Table 6.

Table 6. PCR Thermocycler Program

Step	Temperature	Time
Initial PCR Activation (1 cycle)	94 °C	3 min
PCR (40 cycles)		
Denaturation	94 °C	1 min
Annealing	50 °C	1 min
Extension	72 °C	1 min
Final Extension (1 cycle)	72 °C	7 min
Hold	4 °C	Indefinitely
Product size: 256 bp		

Again, the success of the PCR reactions was initially confirmed by agarose gel electrophoresis (PCR product of 256 bp) in gels composed of 2% agarose with 4 μl of 10

mg/ml ethidium bromide. Gels were typically run at 75 volts for 1 hour. Molecular weight markers were included on the gel.

Reverse Line Blot Hybridization assay for confirmation and genotyping of F+ RNA and F+ DNA coliphages

For rapid confirmation and genotyping of F+ DNA coliphages, a recently developed reverse line blot hybridization assay (RLB) was performed on PCR-amplified products (Vine et al. 2004). For this, 5'-biotinyylated (RT)-PCR products were generated similar to the (RT)-PCR protocols, but with the reverse primers labeled with biotin. Prior to hybridization, the pre-labeled membrane was washed for 5 minutes in 200 ml of 2X SSPE - 0.1% SDS at room temperature. The membrane was assembled in the miniblotter, in a perpendicular position relative to the slots on the lines which contained the probes. Slots were filled with heat-denatured, biotin-labeled PCR product (5 µl of PCR product was diluted in 2X SSPE - 0.1% SDS, heated for 10 minutes at 99 °C and chilled on ice). After hybridization at 48 °C for 60 minutes the membrane was washed twice in 25 ml 2X SSPE - 0.5% SDS for 10 minutes at the hybridization temperature. The membrane was then incubated in 10 ml of streptavidin-peroxidase conjugate (Roche) diluted 1/4000 in 2X SSPE - 0.5% SDS for 45 min. at 42 °C followed by four washes (5 min each) with 2X SSPE - 0.5% SDS at 42 °C and twice for 5 minutes in 2X SSPE at room temperature. After incubating for 1 minute with ECL detection reagent (Amersham) according to the manufacturer's instructions, the membrane was exposed to an X-ray film (Kodak Biomax Light film) for 30 minutes to visualize bound probe. Films were developed in a Konica SRX-101A film developer.

Membrane stripping

After exposure, the membrane was washed four times for 5 minutes in 100 ml 1% SDS at 70 °C on a rocking platform (150 rpm). The SDS was washed away with 2x SSPE and the membrane was then incubated in 20mM EDTA for 15 minutes at room temperature and stored at 4 °C.

Lab Scale Heat Inactivation Experiments on Coliphages

Phage Preparation

F+ RNA phages and F+DNA phage isolates collected from the municipal sewage were divided into two groups, feed isolates and heat-treated isolates. F+ RNA phages were also divided into the two groups detected, group I (MS2) and group III (Qβ). If possible, ten isolates were to be chosen from each group as representative isolates of each sludge type for the heat treatment assay: ten group I feed isolates, ten group I batch (heat-treated) isolates, ten Qβ feed isolates, ten F+ DNA feed isolates, and ten F+ DNA heat-treated isolates. To prepare the isolates, enriched phage tubes were defrosted and centrifuged at top speed (10,000-14,000) for 5 minutes to remove the *E. coli* F_{amp}. After the tubes were centrifuged, the supernatant consisting of phage and TSB was placed in a sterile Eppendorf ® tube. Each isolate was then diluted in TSB until they reached to $10^7 - 10^8$ dilutions and stored at -20 °C until needed.

Obtaining more isolates

To obtain more feed isolates, extra sludge was received from SCWRF. The sludge was similar in type and quality to that of the feed sludge tested in the thermophilic anaerobic

digestion experiments. The sludge was immediately blended in a Waring® blender on high for 1 minute. Serial 10-fold dilutions were then made of the sludge samples in PBS. TSA plates were made again with antibiotics and *E. coli* F_{amp} hosts. Spots of 10 µl of the undiluted sludge and sludge dilutions were pippetted to the TSA plates, allowed to dry, inverted, and incubated overnight at 37 °C. Isolates were then collected from lysis zones, frozen, characterized, and serotyped using the same methods described above.

Heat Trials at 53 °C

The isolates of groups I (MS2) and III (Qβ) F+ RNA and F+ DNA coliphages were exposed to 53 °C in an automated, thermoregulated heat block and titered after 5 and 60 minutes. An Isotemp 125D (Fisher Scientific) thermal heat block was preheated and set at 53 °C. This temperature was selected taking into account the typical heat treatment condition for potential use in thermophilic anaerobic digestion.

A 200 µl volume of each phage (approximately 10^7 PFU ml^{-1}) was distributed into four sterile Eppendorf® tubes. Eight TSA plates were made with the above hosts and antibiotics: two for titering the initial dilutions, two for titering the phages exposed to 5 minutes of heat; two for titering the phages exposed to 60 minutes of heat, and two for titering the phages set at room temperature (unheated controls) for the duration of the experiment.

Each isolate was initially titered using the Spot Titer Culture Assay (Josephs et al. 2004) that will be explained below. Two tubes of each isolate were then placed into the heat block and the time commenced. At the predetermined intervals (of 5 minutes and 60 minutes), one tube of each isolate was removed and cooled in ice for 15 seconds in order to

cool the phages to 40 °C. The surviving bacteriophages were assayed immediately after they were removed at 5 and 60 minute intervals using the Spot Titer Culture Assay.

The pre-selected 5 minute interval was to represent the short term effects of heat on the coliphages. The pre-selected 60 minute interval was to represent long term effects of heat on the coliphages. Lastly, the phages that remained at room temperature for the entirety of the heat inactivation assay were titered. These were to act as a control and indicate any loss of coliphage titer that happens at room temperature during the time of the experiment. The results were expressed as the concentration (pfu ml^{-1}) of viable bacteriophages and plotted against the time.

Preliminary Experiment

Prior to testing the isolates on the heat block, a preliminary experiment was conducted to test how long it took for the test medium in which the coliphages were suspended (TSB) to heat up to 53 °C after sitting at room temperature (25 °C). To do this, 200 µl of room temperature TSB was placed in an Eppendorf® tube. A thermometer tip was then placed into the tube. Once the thermometer was at room temperature, the tube and thermometer were placed into the thermoregulated heat block, which was set at 53 °C. At this point, a timer began and data were collected on the time it took for the temperature of the liquid to rise to 53 °C. It took approximately 1 minute and 30 seconds to raise the temperature to slightly over 50 °C. The amount of additional time needed to raise the temperature the addition 3 °C varied but was generally about 45 seconds.

Additionally, a similar experiment was conducted to see how long it took for the liquid to reach 40 °C after immersing the sample tube in ice immediately after removing it from the heat. It only required about 30 seconds for the liquid to cool to this temperature.

Therefore based on these studies, the timing of heat exposure did not commence until about 1 minute and 30 seconds after the tubes were placed in the thermoregulated heat block. Therefore, these heat inactivation experiment tested the response of the isolates only when they were definitely at thermophilic temperatures of 50 °C or higher (with a maximum of 53 °C in these experiments). Furthermore, the isolates were placed in ice for about 30 seconds after heat exposure, in order to cool them quickly to a temperature at which there would be no rapid inactivation.

Bacteriophage Enumeration Technique: Spot Titer Culture Assay

Ten-fold dilutions were made of each coliphage isolate sample and spotted in duplicate on agar medium-host cell plates. The serial dilutions were made in the following manner. A 10 μl portion of the undiluted phage was first spotted to the TSA plate. Another 10 μl of the undiluted phage was then placed in 90 μl of PBS, vortexed, and a 10 μl of this dilution was spotted in duplicate on the TSA plate using a new pipet tip. This was considered to be 10^{-1} dilution. Immediately after spotting the 10^{-1} dilution on the TSA plates, another 10 μl was taken out of the 10^{-1} dilution tube and placed in another tube containing 90 μl of PBS to make a 10^{-2} dilution. The spotting technique continued to a 10^{-6} dilution. Therefore, each isolate was thus spotted in duplicate initially, after 5 minutes, after 60 minutes, and at the end of the experiment at the following dilutions: undilute, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.

The spots were allowed to dry for 30-60 minutes. The plates were then inverted and incubated overnight at 37 °C. The following morning the phages were enumerated by counting the number of plaques in each 10 μl spot per dilution per sample. Between 1-40 plaque spots are countable and produce the most reproducible results. Coliphage titers were expressed at plaque-forming units per ml.

Methods Comparison

A comparison was made between the spot titer method and double agar layer (DAL) pour plate method on primary effluent and a control coliphage (MS2). The spot titer method was conducted as described above.

For the DAL, 100 x 15 mm tryptic soy agar (TSA) plates (bottom agar) were made with 1.5% bacto agar in TSB the day before the experiment. The day of the experiment, serial 10-fold dilutions were prepared in TSB of the primary effluent samples. A top agar was made consisting of 3 g of TSB and 0.7 g of bacto agar per 100 mls of water. Antibiotics, strep/amp, were added once the media was cooled to ~45 °C. Each dilution was plated in duplicate. Five ml of fresh TSA agar, 100 μl of an overnight culture of *E. coli* F_{amp}, and 100 μl of the sample dilution were added to a 15 ml glass tube. The tubes were then swirled and poured into the Petri dish holding the hardened TSA bottom agar.

For all methods, the plates were allowed to dry, inverted and incubated at the appropriate temperatures. Counts of plaque forming units from the DAL and spot-titer techniques were determined after the incubation period, which was about 8-12 hours. Titers obtained for the DAL and spot-titer technique were compared at each dilution.

Dilution Media Comparison

Additionally a dilution medium comparison was conducted between PBS (Dubeccos), regular PBS, 3% beef broth, and TSB. A group I field isolate and a control (MS2) were chosen for testing. These were then serially 10-fold diluted in all of the described media and spot-titered using the method described above. Each spot was made in duplicate. The spots were allowed to dry, the plates were inverted, and then incubated at 37 °C for 8-12 hours. Titers of the coliphages obtained from the different media were then compared.

Inactivation Rate Kinetics for 53 °C Bench-scale Experiments

The inactivation rate kinetics caused by the heat can be evaluated using the data on phage concentration as a function of time. If each phage is inactivated exponentially (first-order reaction) at a rate unique for that phage, the relationship between concentrations of infectious phage particles (N_A, N_B) and time (t) is:

$$dN_A/dt = -k_1 N_A \qquad (1)$$

$$dN_B/dt = -k_2 N_B \qquad (2)$$

where k_i and k_2 are proportionality constants and dN_i/dt are rates of changes of concentrations with time. A first-order reaction is one in which the probability of being inactivated is related exponentially to the time according to equations 1 and 2 above (Hiatt 1964).

However, viral inactivation is not always simple in form. The data for thermal inactivation may appear as two lines of different slopes, indicating different decay rates (k_i) are present. This may provide evidence for the participation of two different reaction

mechanisms, heterogeneity (or multiple populations) within the phage mixture, or virus aggregation. This state can be evaluated using the following equations.

Integrating Eqs. (1) and (2) between limits yields:

$$N_A = N_A^0 \exp^{(-k1t)} \quad (3)$$

$$N_B = N_B^0 \exp^{(-k2t)} \quad (4)$$

In which N_i^0 are the concentrations at the beginning of the thermal treatment, and Ni are the concentrations after time t has elapsed.

So, the survival of virus particles over time can be represented by:

$$N = N_A + N_B \quad (5)$$

$$N = N_A^0 \exp^{(-k1t)} + N_B^0 \exp^{(-k2t)} \quad (6)$$

The initial viral population is expressed as:

$$N0 = N_A^0 + N_B^0 \quad (7)$$

$$1 = N_A^0/N^0 + N_B^0/N^0 \quad (8)$$

and defining:

$$\gamma = N_B^0/N^0 \quad (9)$$

and

$$(1-\gamma) = N_A^0/N^0 \quad (10)$$

and the survival ratio for the phages may be written as:

$$N/N^0 = (1-\gamma)\exp(^{-k1t}) + \gamma\exp(^{-k2t}) \quad (11)$$

in which fraction $(1-\gamma)$ of the phage is inactivated at the rate constant k_1 and fraction γ is inactivated at the rate constant k_2. Consequently, a survival curve is the sum of two linear components which intercept at $(1-\gamma)$ and γ respectively.

From the experimental data of phage survival at 53 °C heat treatments, N/N^0 ratios were calculated and plotted against time. These data were fitted by following the model previously proposed (Hiatt 1964 and Quiberoni et al. 2003) and with the help of Prof. Douglas Crawford-Brown. The first-order kinetic line and the "two population" line were fitted to the data in order to illustrate which type of kinetic model best described in the inactivation of the phages by heat. These graphs were then used to make comparisons between the phages to help understand the tailing effect witnessed in the thermophilic anaerobic digestion inactivation experiments. The graphs were created using the computer program Excel.

Statistical Analysis of the Log Inactivation

Analysis of variance (ANOVA) and post-tests were performed on the isolate data obtained after they were exposed to heat for 60 minutes, with the computer program INSTAT to generate the significance values. An ordinary ANOVA compares the means of three or more groups. The null hypothesis is that all column means are equal and INSTAT reports the p-value testing this null hypothesis. The ANOVA test assumes that the data are randomly sampled from larger populations (or at least are representative of those populations), that each values was obtained independently of the others, that the data are sampled from populations that follow a Gaussisan distribution and that the standard deviations of these populations are all equal.

When a significant result was obtained in ANOVA, a Tukey-Kramer Multi-comparison Post Test was used to narrow down which columns were significantly different

from other columns. For every pair of columns, the difference between means and p-values was reported.

IV RESULTS

Thermophilic Anaerobic Digestion Experiments

Male-Specific Coliphage Removals by treatment in a Continuous Digester

A summary of the physical/chemical characteristics of the feed sludges and effluent biosolids is provided in Aitken et al. (submitted). Although temperature is believed to be the primary factor responsible for the inactivation of pathogens and indicator organisms in high-temperature processes such as thermophilic anaerobic digestion, other physical and chemical characteristics of the sludge that may also influence the inactivation of these organisms include pH, concentrations of protonated (non-ionized) organic acids, and free NH_3 (non-ionized ammonia). The reduction of any pathogen or indicator organism in the continuous digester is reflective of the overall conditions in the digester. Because the continuous digester was completely-mixed and essentially continuous flow, the effluent concentrations of potentially relevant parameters reflect the conditions to which the organisms were exposed in the reactor.

Additionally, the residence time in the continuous-flow reactor was between 4.0 and 4.3 days at 55 °C, for which two distinct operating periods reported. The residence time for the remainder of the operating conditions was between 5.3 and 6.0 days (Aitken et al. submitted). These residence times were selected to be conservatively short for single-stage thermophilic anaerobic digestion. Because the continuous reactor was operated at a shorter residence time at 55 °C than the other operating conditions, removals at 55 °C cannot be

compared directly on the basis of equal residence time to removals under the other conditions.

In Aitken et al. (submitted), no infectious poliovirus that had been seeded into the digester feed sludge was found in the digester effluent at any of the operating conditions. At least a three-log removal across the reactor was observed under all operating conditions except during the first period of operation at 55 °C, for which a substantially lower concentration had been spiked into the feed sludge and hence for which only a lower maximum $\log_{10}$ reduction could be observed. Viable *Salmonella* were found in the digester effluent in one sample collected during operation at 53 °C with the SCWRF sludge, but viable *Salmonella* were not found in the effluent under any other operating condition. The extent of fecal coliform reduction by the continuous digester appeared to increase with increasing temperature, but the ability of the continuous digester to achieve an effluent concentration below 1,000 MPN/g TS (the Class A limit) depended on the fecal coliform concentration in the feed sludge. Both effluent samples collected during operation at 55 °C had fecal coliform concentrations greater than the Class A limit. However, it should be kept in mind that the continuous digester was operated at a shorter residence time at 55 °C than at the other operating conditions. *Clostridium perfringens* spores were not reduced in the continuous reactor under any operating conditions. Reduction of somatic coliphages was generally less than one-log and there was no apparent trend with temperature. For this reason, neither of these organisms would be good indicators of thermal inactivation of pathogens over the range of temperatures evaluated in this study. This is because they were not reduced by sufficient amounts to documents the 2- and 3-$\log_{10}$ reductions required for helminthes and viral pathogens by a PFRP process.

Data on reductions of male-specific coliphages by the continuous flow thermophilic digester illustrated more promise as an indicator of pathogen reductions for the temperatures studied. These data are summarized in Table 7. Male-specific coliphages were less abundant in each sludge than the somatic coliphages (Aitken et al, submitted), although the concentration was at or above 4.5 $\log_{10}$ MPN/g TS in some of the SCWRF sludge samples. The reduction of male-specific coliphages in the continuous digester appeared to be related to temperature. Greater than two-$\log_{10}$ reduction was observed at 55 °C, from 0.9 to 1.6 $\log_{10}$ removal was observed at 53 °C for all three sludges, and no significant reduction was observed at 51 °C. The concentration of coliphages in the feed and treated sludges was evaluated statistically using a two-tailed t-test with the assumption of equal variances. There was a statistically significant difference in the phage $\log_{10}$ concentration between the "feed" and "effluent" for each sludge sample at 53 °C and 55 °C, but not at 51 °C. Again, it should be noted that the residence times used during the two periods of operation at 55 °C were lower than used during the other operating periods. The concentration ($\log_{10}$/g TS) and $\log_{10}$ reduction of each feed sample in the continuous flow reactor can be found in Appendix 1.

Table 7. Concentration ($\log_{10}$/g TS) and Reductions of Male-Specific Coliphages by Treatment in the Continuous Digester

	Concentration ($\log_{10}$/g TS)		
Operating Condition	**Feed**	**Effluent**	**$\log_{10}$ Reduction**
SCWRF-55A	4.59 ± 0.47 (3)	2.23 ± 0.28 (2)	2.36 ± 0.55
SCWRF-55B	4.44 (1)	1.84 (1)	2.60
SCWRF-53	3.31 ± 0.41 (5)	2.17 ± 0.04 (4)	1.14 ± 0.42
SCWRF-51	3.37 ± 0.39 (4)	3.11 ± 1.10 (2)	NR [a]
OWASA-53	1.21 ± 0.68 (4)	0.35 ± 1.12 (2)	0.86 ± 1.31 [b]
WLSSD-53	2.22 ± 0.12 (4)	0.65 ± 0.06	1.57 ± 0.13

[a] NR = no removal (no significant difference between feed and effluent concentrations).

[b] Although the difference between the mean feed and effluent concentrations is not statistically significant ($p > 0.05$), one of the effluent concentrations was lower than three of the four influent concentrations and the other was lower than all four concentrations. The higher of the effluent concentrations (1.14 $\log_{10}$) was

lower than the concentration in both feed samples obtained before that effluent sample was collected (1.72 $\log_{10}$ and 1.48 $\log_{10}$). The relatively low mean and high standard deviation for the feed concentration are a result of one very low value (0.20 $\log_{10}$)

Batch Treatment of Effluent Biosolids from the Continuous Digester

For each operating condition two tests were conducted in which the effluent from the continuous digester was treated in a batch reactor for another eight to 24 hours. The effluent from the continuous flow reactor was equivalent to time 0 in the batch reactor and further reductions of microorganisms in the same material were measured. Removal of male-specific coliphages on the order of 0.5 to greater than one- or even 1.5 $\log_{10}$/g TS occurred over eight to nine hours of batch treatment. However the extent of coliphage reduction did not follow any trend of increased reduction with increased thermophilic temperature.

Table 8. Concentrations (Log10/g TS) and Reductions of Male-Specific Coliphages Treatment in the Batch Reactor

		Concentration at Indicated Time (Log_{10}/g TS)								
Condition	**Date**	**0**	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**8 hr**	**9 hr**	**24 hr**	**Total Removal**
SCWRF-55 °C	9/7/2002	2.03	< 1.95	< 1.82		< 1.69	< 1.61			>0.42
	9/14/2002	2.48	2.26	2.57		1.93	2.05			0.43
SCWRF-53 °C	1/18/2003	2.18	1.93		1.01			1.16	1.79	0.39
	1/25/2003	2.2	1.62		1.48			1.8	1.6	0.6
SCWRF-51 °C	3/15/2003	3.89	3.92		3.65			2.53	2.54	1.35
	3/22/2003	2.33	1.35		1.25			1.27	1.26	1.07
OWASA-53 °C	4/5/2003	1.14	< 0.85		1.14			< 0.92	< 1.09	>0.05
	4/12/2003	-0.44	< -0.39		< -0.36			-0.25	0.05	-0.49
WLSSD-53 °C	4/26/2003	0.61	< 0.61		0.24			< -0.51	< -0.51	>0.1
	5/3/2003	0.69	0.21		0.17			0.25	-0.42	1.11

Empty cells in the table indicate that a sample was not collected at those time points. All "less than" values represent samples with concentrations below the lower detection limits. Some of the $\log_{10}$ concentrations that are "less than" values make it difficult to

estimate actual $\log_{10}$ reductions. The actual $\log_{10}$ reductions could be much greater than indicated by the "less than" value. Also, in some cases apparent increases in concentration occur with time, particularly for those samples collected at 24 hours after the batch test was initiated. These increases are largely due to reductions in total solids concentrations which are caused by a breakdown of total solids in the batch reactor with time. All data at time 0 represent effluent concentration from the continuous digester on the date of the batch test.

Results of Inactivation Rate Experiments

The inactivation kinetics of coliphages was further evaluated under batch conditions to quantify inactivation rates as a function of temperature. These tests differ from the batch tests discussed above in that the organisms were spiked directly into the batch reactor, which contained biosolids removed from the continuous digester. These tests were designed to minimize the change in temperature caused by spiking in the organisms. Addition of the spiking material at the beginning of each test led to a predictable drop in temperature of 0.3 to 0.4 °C, but the temperature returned to within 0.1 °C of the target temperature within five to eight minutes. Profiles of the temperature shortly before and after adding the spiking material at each temperature evaluated are provided elsewhere (Aitken et al., submitted).

Results for inactivation of male-specific coliphages in the batch reactor over time suggest that there might be a heat-resistant fraction. Reductions of male-specific coliphages over 24 hours were more extensive than were observed with somatic coliphages except at 55 °C (Aitken et al., submitted), for which a negligible quantity of male-specific coliphages were added to the batch reactor in the spiking material. In that experiment the majority of the male-specific coliphages present at time 0 were those remaining in the effluent from the

continuous digester. Changes in concentrations of male-specific coliphage over time at 51 °C and 53 °C are shown in Figure 4. These figures illustrate the apparent existence of at least one fraction of the F+ coliphage population that is heat-sensitive and one that is heat-resistant. About 2 to 3 $\log_{10}$ of the initial coliphages were inactivated relatively rapidly, within 2 hours, and the remaining ones are very persistent, with little further reduction in coliphage concentration for the duration of the 24-hour experiment.

If inactivation of the heat-sensitive fraction of the male-specific coliphages follows first-order kinetics and we assume there are two populations, then the following two population equation can be used to estimate the rate coefficient at a given temperature:

$$C = C_r + (C_0 - C_r)e^{-kt} \quad (1)$$

where C is the total concentration of coliphages, C_0 is the concentration at time 0, C_r is the concentration of heat-resistant coliphages, and k is the inactivation rate coefficient of the heat sensitive coliphages. The inactivation rate of the heat-resistant population was assumed to be negligible. Fits to this equation in which both C_r and k were the fitting parameters are shown in Figure 4 along with the experimental data, which can be found in Appendix 2. Fitted parameter values are summarized in Table 9 There was no difference in initial inactivation rate (the heat-sensitive fraction) between the two temperatures evaluated (which can also be observed by comparing the curves in Figures 4(a) and 4(b) and by looking at the k values in Table 9 (Aitken et al. 2003).

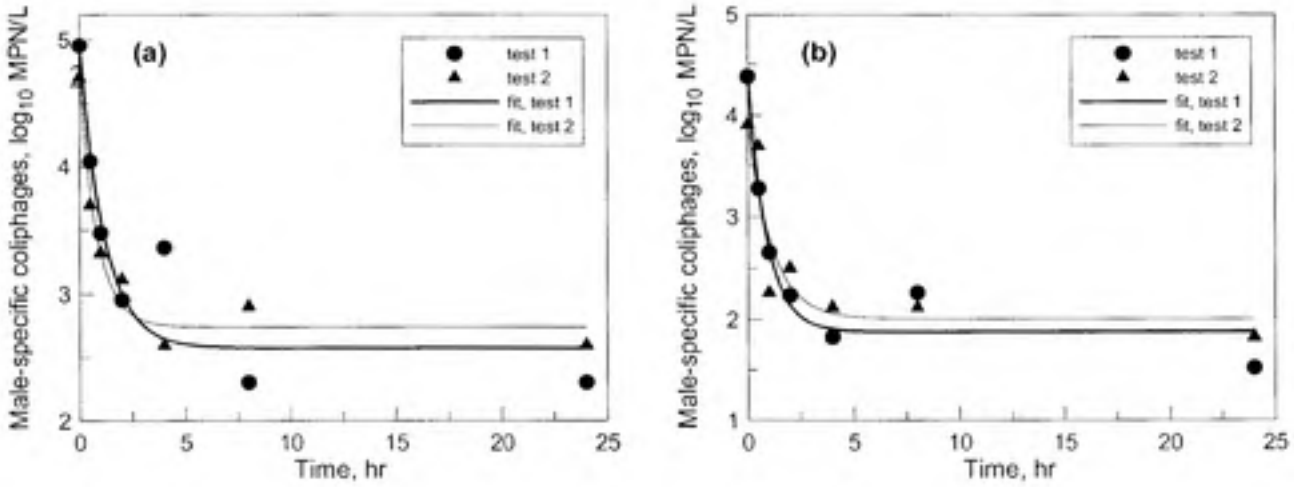


Figure 4. Concentrations of Male-Specific Coliphages as a Function of Time in Inactivation Rate Experiments at (a) 51 °C and (b) 53 °C.

The lines represent fits to the data by non-linear regression of equation 1 (two-parameter fits for C_r and k).

Table 9. Best-fit Parameters for Inactivation of Male-specific Coliphages

Temperature (°C)	Test	C_r ($\log_{10}$ /L)	k (hr^{-1})	r^2
51	1	2.6 ± 0.6	0.9 ± 0.8	0.88
	2	2.7 ± 0.2	1.2 ± 0.6	0.96
53	1	1.9 ± 0.4	1.1 ± 0.6	0.95
	2	2.0 ± 0.5	0.9 ± 0.9	0.86

There appears to be a difference in the fraction of the total coliphages resistant to heat at the two different temperatures. The C_r values and examination of the graphs in Figure 4 indicate more phages were heat resistant in the 53 °C experiment. This may have occurred because different batches of biosolids were used in these experiments with more heat resistant F+ coliphages having been present in the background feed sludge used in the 51 °C experiments compared to those in the 53 °C experiment.

Male-Specific Coliphage Characterization

RNAse and Genotyping Results

Because the inactivation rate experiments suggested a difference in heat resistance between two or more populations of male-specific coliphages, a collection of isolates was taken from the feed sludge and batch reactor for characterization to evaluate if the resistance was characteristic of the F+ RNA coliphages or the F+ DNA coliphages. These isolates were collected from samples that had been exposed to 51 °C and 53 °C and were collected as isolates of plaques from the MPN spot plate analysis of sludge samples from the feed and effluent of the continuous flow digester and batch reactor. Prior to testing, these isolates were regrown by an enrichment assay.

An RNAse test was conducted to evaluate any trend in presence of F+ RNA or F+ DNA coliphages in the feed and batch reactor samples. A spot-plate RNAse assay was run of samples that contained about 10-100 pfu per spotted volume. RNAse was added to the agar media to suppress the growth of F+ RNA coliphages. Any growth of phage in a lysis zone was taken to indicate the presence of an F+ DNA. A comparison agar plate without RNAse was also spotted with the same isolates. Both F+ RNA and F+ DNA coliphages isolates were to grow on this plate.

Based on the RNAse testing results, it was initially mistakenly concluded that there was no apparent trend of survival difference between the F+ RNA and the F+ DNA phage isolates. However, after further confirming the identity of the isolates using PCR, it was found this initial procedure to identify F+ RNA and F+ DNA coliphages was not reliable. All RNAse-characterized F+ DNA isolates were genotyped by PCR to confirm their identity. Of 66 phages initially characterized as F+ DNA, only 21 were positively F+ DNA phages using PCR, 35 were confirmed as F+ RNA phages using RT-PCR (27 group I, 8 group I-

group III mix), and 10 phages needing further analysis for conclusive identification, such as by re-analysis using nucleic acid methods or by electron microscopy.

Table 10 illustrates the true identification of the phage types (F+ RNA or F+ DNA) found in the feed sludge samples and the batch sludge samples using PCR and RT-PCR. As shown, there are approximately similar percentages of F+ DNA and F+ RNA coliphage isolates in the feed sludge samples, about 54 and 43 percent, respectively.

In the batch reactor samples, however, the majority of the batch reactor isolates, 73 of 84 or 87% were confirmed as F+ RNA coliphages. There was only one F+ DNA isolate found, and it was found at sample time 8 hours in the batch reactor. The presence of this F+ DNA phage at the 8-hour sampling time suggests that at least some of the F+ DNA phages may be relatively heat resistant. However, it is puzzling that there were not more F+ DNA phages represented in the batch reactor samples. The presence of mostly F+ RNA coliphages in the batch reactor samples suggests that these phages may be relatively heat-resistant.

Table 10. F+ DNA and F+ RNA Isolates found in Feed and Batch Samples

Feed Isolates (number and percentages)			Batch Isolates (number and percentages)		
F+ RNA	16	43%	F+ RNA	73	87%
F+ DNA	20	54%	F+ DNA	1	1.2%
Unidentified	1	2.7%	Unidentified	10	12%
Total	37		Total	84	

Serological Typing of F+ RNA Coliphage Isolates

F+ RNA coliphage isolates were further characterized by subdividing into one of five groups by spot neutralization tests with antisera raised in rabbits against prototypical viruses of each group. The groups were: I (MS2), II (GA), III (Qβ), IV (SP), IVa (FI). The latter two subgroups of IV are closely related bacteriophages. Only group I and group III isolates were found in the feed and batch samples. Furthermore, there was a predominance of group I

isolates found in the thermophilic treatment samples taken from the batch reactor, with 56% group I and 38% group III. These data suggest that the group I F+ RNA coliphages may be the relatively heat-resistant fraction because they are the predominant F+ RNA coliphage group detected after thermophilic treatment in the batch reactor. Group III F+ RNA coliphage isolates were found in the batch reactor treated samples but only at the earliest sample times of 0, 0.5 and 1 hours. Again, time 0 is equivalent to the effluent of the continuous-flow reactor. These data provide some evidence that group III F+ RNA coliphages can survive the conditions in the continuous-flow reactor. However, there is evidence for their survival for only up to one hour in the batch reactor at the thermophilic temperatures tested. Table 11 shows the numbers of F+ RNA coliphage isolates of different groups in both the feed and batch samples and their percentages relative to the total number of isolates. A more detailed table on the sources and properties of these isolates can be found in the Appendix 3.

Table 11. F+ RNA Serotypes found in Feed and Batch Samples

F+RNA Feed Isolates (number and percentages)			F+RNA Batch Isolates (number and percentages)		
Group I (MS2)	9	56%	Group I (MS2)	66	90%
Group III (Qβ)	6	38%	Group III (Qβ)	0	0.00%
Both (I and III); mixture	1	6.2%	Both (I and III); mixture	7	9.6%
Total	16		Total	73	

It should be noted that 38 of the 84 batch isolates (45%) were taken from biosolids spiked with organisms for the kinetic inactivation experiments in the batch reactor. This spike mixture of coliphages was originally concentrated from raw wastewater collected at the wastewater treatment plant in Beaufort, NC. Unfortunately it was not characterized for the concentrations of the various coliphages. It is likely that this concentrated spike of sludges did not contain all representative groups of F+ RNA and F+ DNA coliphages. Therefore,

without having such data on the characteristics of these F+ coliphages, it is not possible to reliably identify the most resistant phages.

However, there were 46 other F+ coliphage isolates characterized from the non-spiked biosolids, and they provide similar results regarding the presence of different nucleic acid types and groups within each nucleic acid type. As shown in Table 11, there was a preponderance of group I coliphages present in the batch reactor samples (36 of 46 samples were group I and 10 were unidentifiable). Because it was already established that the there were similar concentrations of F+ RNA and F+ DNA in the feed samples, these results provide evidence that the F+ RNA Type I were more resistant than other F+ RNA coliphages to the thermal effects in the batch thermophilic anaerobic digester.

PCR-RLB typing of F+ DNA coliphage isolates

Because F+ DNA coliphages comprised a substantial proportion of the F+ coliphages in the feed sludge to the batch reactor and at least one F+ DNA coliphage was found in the batch treated sludge, it was of interest to genetically characterize at least some of these isolates. This characterization was done to determine if any genetic type or group of F+ DNA coliphage appeared to be more heat resistant or predominate in the biosolids than other genetic types. A sample of 7 F+ DNA coliphage isolates were characterized using reverse line blot hybridization. The results are summarized in Table 12. There was no particular F+ DNA phage type or group that persisted or appeared more frequently than the other groups. However, the numbers of isolates are few (7) and therefore, it is not possible to reliably determine if there were differences in thermal resistance among the different genetic groups of F+ DNA coliphages. It is possible that particular genetic groups of F+ DNA coliphages

are more or less heat-resistant than other groups, but further studies to characterize the group identities of more isolates would be needed to determine this.

Table 12. F+ DNA Genotypes found in Feed Samples via RLB-Hybridization

Genetic Group of F+ DNA isolates	Number of isolates
CH	2
Fd	2
CH-M13	2
Fd-M13	1

Lab Scale Thermal Inactivation Experiments on F+ Coliphage Isolates

Isolates that were collected from the feed sludges and the batch reactor at varying times at 51 °C and 53 °C were used for the lab-scale thermal inactivation experiments. Their collection method is further explained above and in the Methods and Materials section. After isolates were collected, they were enriched in tryptic soy broth with F_{amp} host overnight and then frozen until ready for use. Once thawed, they were centrifuged for 15 minutes to separate the coliphages from the *E. coli*. The supernatant was then placed in another sterile Eppendorf® tube. This process was intended to provide pure cultures of individual coliphages. Again, tryptic soy broth was the medium used for the dilutions made for the spot titer assay.

The results of the inactivation rate experiments in the batch digester and the characterizations of the different F+ coliphages suggested that there were differences in the thermal resistance properties of the different male-specific coliphage groups. Therefore, further lab scale inactivation experiments on these F+ coliphage isolates were conducted at 53 °C and exposure times of 5 and 60 minutes.

Data on the extent of inactivation of male-specific coliphages suspended in tryptic soy broth after 60 minutes of exposure to the thermophilic temperature of 53 °C or to room temperature (about 25 °C) are summarized in the Table 13 below as log_{10} reductions. Because there were unexplained non-specific reductions of these coliphages in aliquots of the same samples held at room temperature and at 53 °C, the log_{10} inactivations due only to the differences in temperature (by subtracting the log_{10} reduction at room temperature from the total reduction ay 60 °C) are also shown. The raw data for titers and log_{10} reductions of each isolate can be found in Appendix 5. The log_{10} reductions and statistical analyses of each coliphage group (F + DNA and F+ RNA group I and III) can be found in Appendix 5.

Table 13. Male-Specific Coliphage Log_{10} Reductions in Lab Scale Inactivation Experiments

Isolate Type and # of Samples	Inactivation (log_{10}) after 60 Minutes at 53 °C	Inactivation (log_{10}) after 60 Minutes at room Temperature (~25 °C)	Inactivation (log_{10}) Attributable to Temperature Effect (Log_{10} reduction difference; Log_{10} 53 °C – Log_{10} Room Temperature [c]
Group I Feed (9)	1.5 (+/- 0.51)	0.76 (+/- 0.53)	0.76 (+/- 0.33)
Group I Batch (13)	1.7 (+/- 0.55	1.1 (+/- 0.49)	0.65 (+/- 0.31)
MS2 Control (5)	1.1 (+/- 0.62)	0.59 (+/- 0.46)	0.54 (+/- 0.72)
Group III Feed (10)	3.8 (+/- 1.49) [a]	0.82 (+/- 0.46)	3.0 (+/- 1.31)
QB Control (8)	4.5 (+/- 1.09) [a]	0.22 (+/- 0.56)	4.3 (+/-1.01)
F+DNA Feed (13) [b]	0.50 (+/- 0.77)	0.33 (+/- 0.48)	0.17 (+/- 0.62)
F+DNA Batch (1) [b]	0.18	0.2	-0.02
F+DNA ATCC (3)	0.21 (+/- 0.29)	0.06 (+/- 0.15)	0.16 (+/- 0.25)

[a] Some isolate numbers are based on less than values (5 in the feed and 2 of the control tests). These values probably underestimate the actual heat sensitivity of these isolates

[b] Only one F+ DNA coliphage was present in the batch samples.

[c] mean +/- standard deviations

The log_{10} reduction data of individual isolates is graphed in the three Figures 5-7. It is apparent from Figure 5 and 7 that the group I F+ RNA coliphages and the F+ DNA

coliphages are inactivated much less than the group III F+ RNA coliphages. Additionally, while some coliphages isolate groups appear variable in Figure 5 and 6, they have more consistent responses within a major group (F+ RNA group I and III or F+ DNA) than between groups after the inactivation occurring at room temperature has been accounted for.

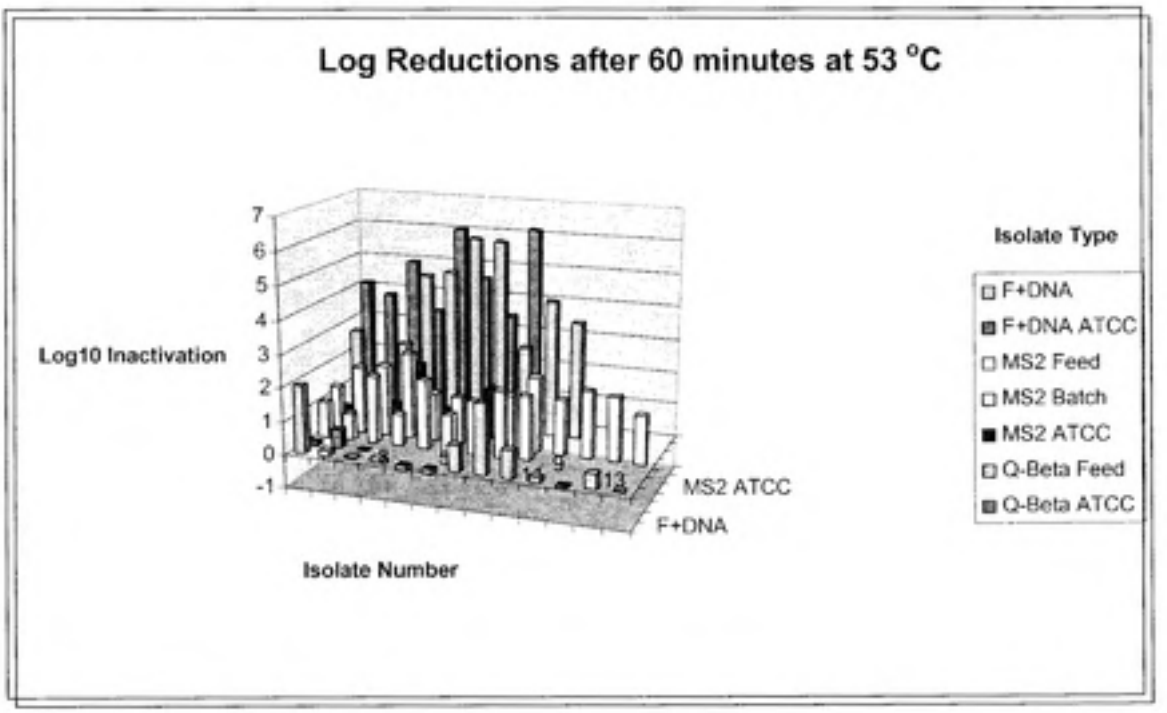


Figure 5. Log_{10} Reductions of Male-Specific Coliphage Isolates after 60 minutes at 53 °C

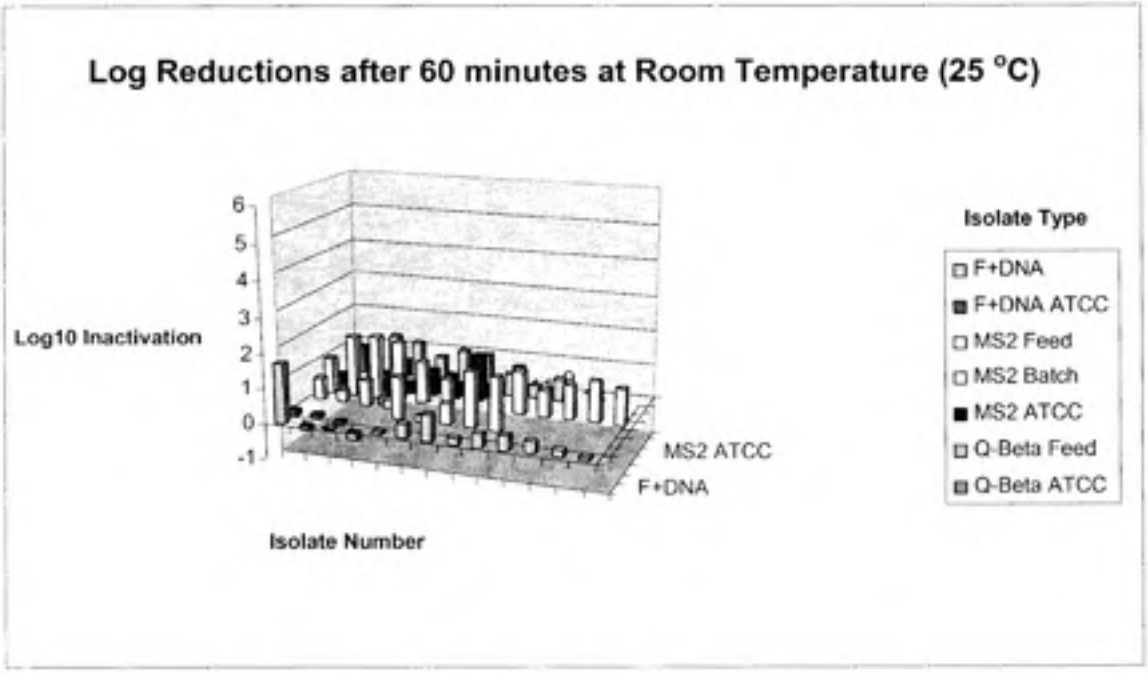


Figure 6. Log_{10} Reductions of Male-Specific Coliphage Isolates after 60 minutes at Room Temperature

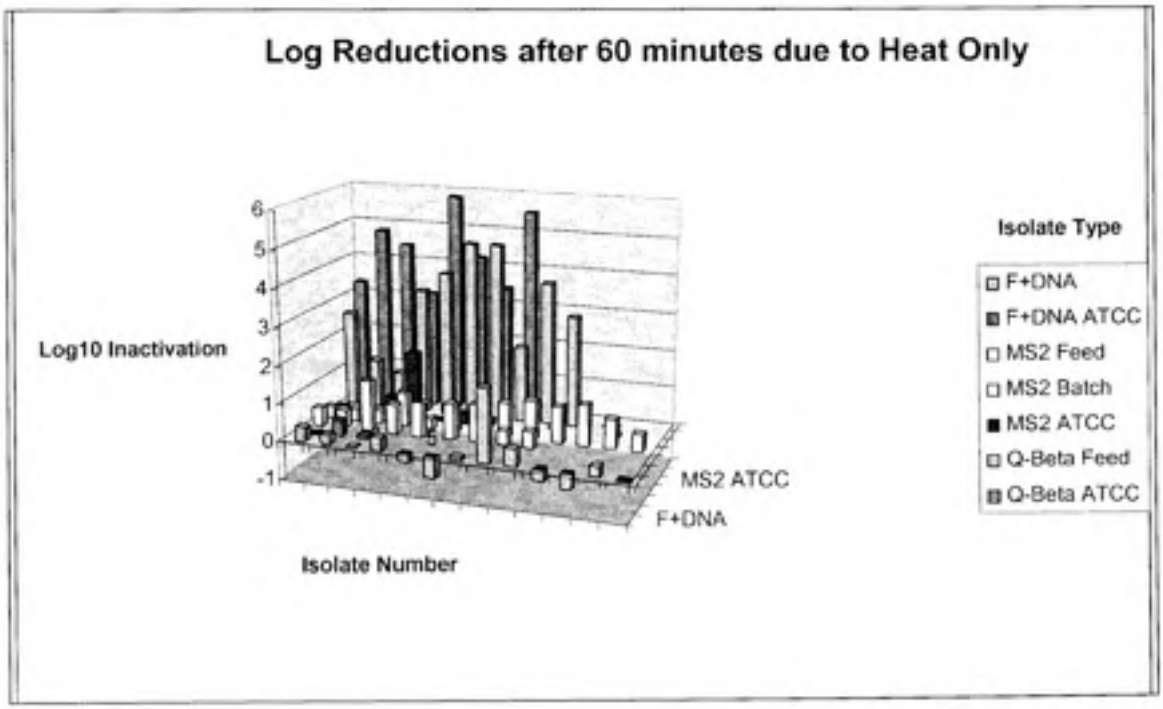


Figure 7. Log_{10} Reductions of Male-Specific Coliphage Isolates Due to Heat at 53 °C Only

An ANOVA statistical analysis was performed on the seven categories of F+ coliphages for their $\log_{10}$ "inactivation attributable to heat at 53 °C" reductions. The $\log_{10}$ reductions of the group III isolates were clearly dependent on temperature, as shown by the inactivation differences between the group III isolates and the group I isolates and between the group III isolates and the F+DNA isolates. Both the group III field isolates (feed) and group III ($Q\beta$) lab strains showed the greatest log reductions. The $\log_{10}$ reductions of the seven categories differed significantly (ANOVA, $P < .0001$). Further, a Tukey-Kramer post test revealed that group III feed and controls coliphages were both significantly different than the group I coliphages (feed, batch and control) and the F+ DNA coliphages (field and control). The group I (feed, batch and control) and the F+ DNA (field and control) coliphages were not shown to be significantly different ($P > 0.05$) from each other. These findings indicate that there are different heat sensitivities among the F+ coliphage groups, with greater sensitivity of the F+ RNA group III coliphages than either the F+ RNA group I coliphages or the F+ DNA coliphages.

It should be noted that seven group III isolates were not detectable after 60 minutes of exposure at 53 °C. That is, these coliphages were reduced to below the detection limit of the assay method, based on the sample size analyzed. To calculate a "less than" numeric value for this non-detect result it was assumed that one phage was present in the total sample volume assayed. Due to the "censored" data of these detection limit results, the $\log_{10}$ reduction results for the group III isolates (feed and control) probably underestimate the actual heat sensitivity of these phages. Because larger sample volumes were not assayed, the actual rate and extent of inactivation is not reliably quantified, and it is not apparent how much inactivation actually occurred for this coliphage group.

Additionally, the $\log_{10}$ inactivation of each isolate was graphed to illustrate if the inactivation followed first-order rate kinetics for a single population or a two-population model. Graphs of all isolates can be found in Appendix 7. The best-fit parameters for the inactivation kinetics, of each isolate can be found in Appendix 8. As examples, two graphs, each with different inactivation kinetics are shown below. One graph depicts inactivation following first-order kinetics (Figure 8 group I Feed (4)) and the other graph follows a two population model (Figure 9 group III Feed (4)). If more data points representing additional sampling times had been taken, the kinetics for the rate and extent of inactivation could be presented with greater certainty. Some of the group III coliphages for which there were no detectable viruses at 60 minutes show a 2 population die-off. The downward pointing white arrow indicates this. These isolates may actually be inactivated with first-order kinetics, but the inability to detect the virus at the 60-minute time point makes it impossible to document such kinetics. In this case, more data points for additional sampling times before 50 minutes and the analysis of greater sample volumes are needed to more accurately quantify the inactivation kinetics

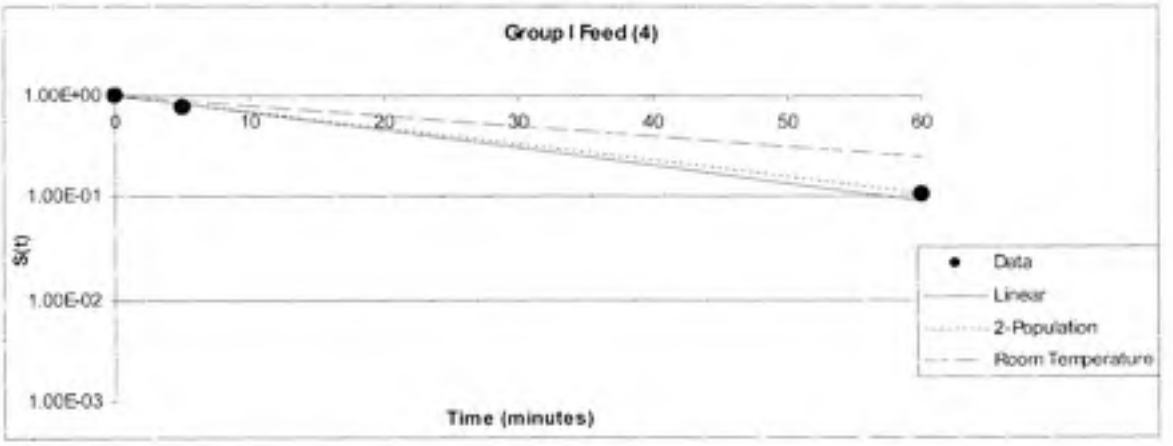
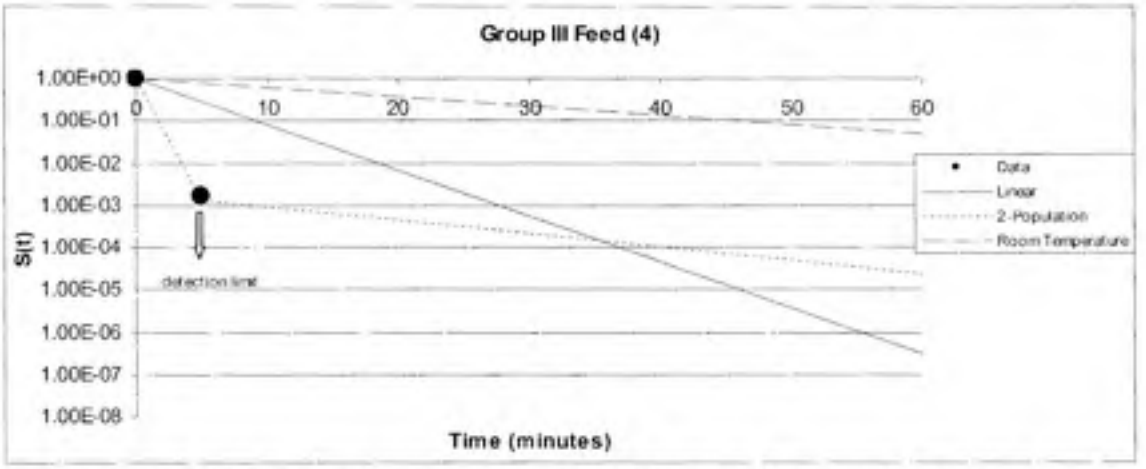


Figure 8. Inactivation of Group I (MS2) F+ RNA Coliphage Feed Isolate Illustrating First-Order Kinetics



*The downward pointing arrow indicates that the detection limit was after 5 minutes and there was no detectable phage after 60 minutes. The dotted line indicates the graph progression if 50 pfu/ml were present at after 60 minutes. However, this is not a particularly useful measure because it underestimates the inactivation after 60 minutes.

Figure 9. Inactivation of Group III (Qβ) F+ RNA Coliphage Feed Isolate

The larger-dash broken line in the inactivation graphs illustrates the log inactivation of coliphages kept at room temperature. These phages were initially kept as a positive control sample for the duration of the experiment and were not expected to demonstrate appreciable loss of infectivity titer. However, as shown in the Table 13 and Figure 9 above, there were variable amounts of coliphage titer loss. The inactivation of the coliphage control samples at room temperature was as great as 1.81 $\log_{10}$. An ANOVA statistical test was performed on the $\log_{10}$ reduction to understand if the there were statistically significant differences in the rate and extent of inactivation at room temperature among the different coliphage groups. The ANOVA test resulted in a P-value < 0.004, which is statistically significant. The Tukey-Kramer post test revealed that the variation among coliphage group means was significantly greater than expected by chance for group I batch versus group III (Qβ) control; group I batch versus F+ DNA isolates; and group I batch versus F+ DNA controls.

Reasons for the loss of infectivity of F+ coliphages at room temperature are unknown. It has been reported that the media can contribute to bacteriophage inactivation when exposed to physical or chemical stress (Adams 1959). However, the media used, tryptic soy broth, is a nutrient medium also used as a diluent and elution solution that is thought to prevent virus adsorption to surfaces such as sample tubes and to protect against inactivation. Additionally, the Eppendorf® tubes used to hold samples during heat exposure also prevent the viruses from sticking to the surfaces of the tube. It is possible that the loss of coliphage titer in samples held at room temperature is transient and potentially reversible, such as caused by aggregation. While TSB is not considered a major cause of aggregation, no attempt was made to determine if reversible aggregation had occurred.

A limited comparison study was conducted among dilution media and between two coliphage enumeration methods to understand if these were possible reasons for the loss of coliphage titer at room temperature. Regular PBS, PBS (Dubeccos), TSB, and 3% beef extract were compared in the dilution media comparison test. The dilution media did not appear to affect the counts for the spot-titer method (Appendix 8). Additionally the spot titer method and the DAL were equal at the 95% significance level for two of the three samples taken from primary effluent (Appendix 9). This information is discussed to a greater extent elsewhere (Josephs et al. 2004). Because the sample size of these comparison tests are small, further studies would be needed to specifically investigate this and other possible causes of the loss of coliphage titer at room temperature.

It is of interest to examine the extent of variability of the responses of different coliphage isolates of a particular genetic type or subgroup. The graphs below provide a visual presentation of the inactivation of the coliphage isolates after 60 minutes of exposure

at 53 °C. These graphs do not specifically show the amount of loss of infectivity at room temperature, but rather present the unadjusted, raw inactivation data of the heated sample. Each sludge feed, batch reactor, and positive control isolate is included in the graph of their respective group: F+ RNA group I, F+ RNA group III, or F+ DNA. The sludge feed isolates are shown with a dotted line, batch reactor isolates by a dashed line, and the positive controls by a solid line.

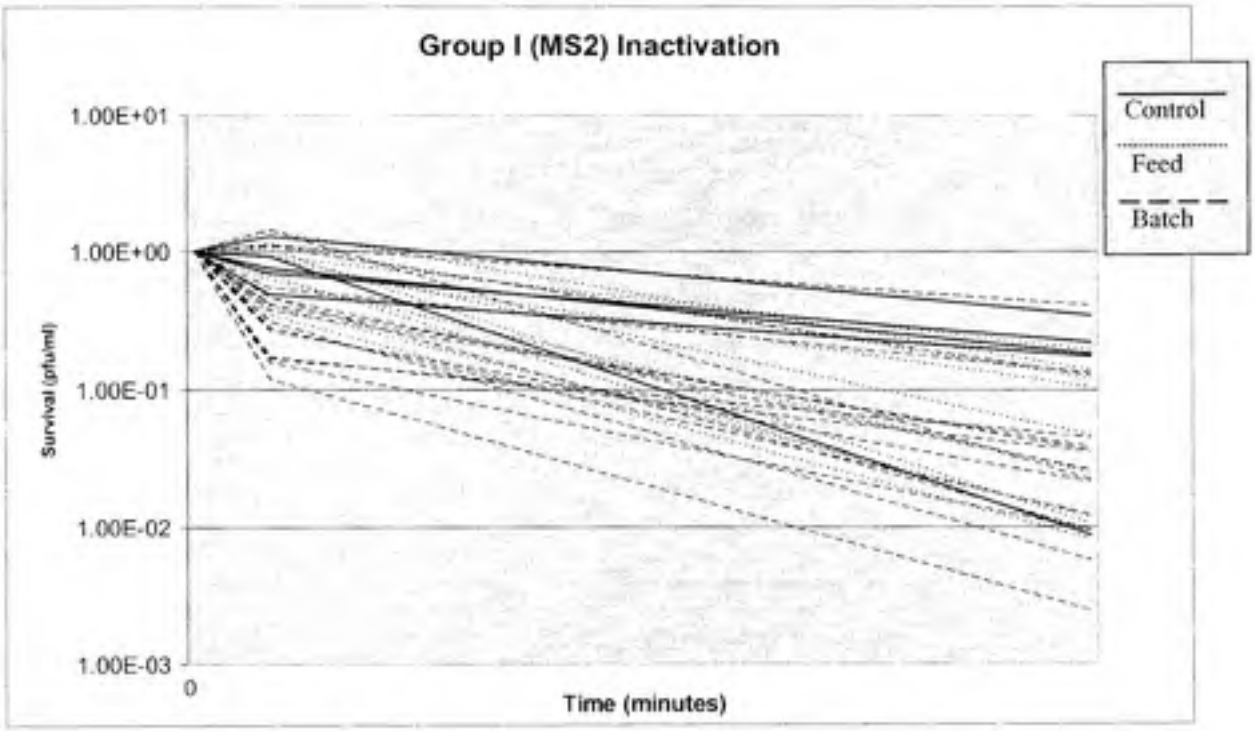


Figure 10. Inactivation Summary Group I (MS2) Feed, Batch, and Control Isolates at 53 °C for 5 and 60 minutes

As shown in Figure 10, the group I feed sludge isolates were inactivated by 0.82-2.1 $\log_{10}$ with a mean reduction of 1.5 $\log_{10}$ and 95% confidence level of ±0.51 $\log_{10}$; group I batch reactor isolates were inactivated by 0.39-2.6 $\log_{10}$ with mean reduction of 1.7 $\log_{10}$ and 95% confidence level of ±0.55 $\log_{10}$; and the group I (MS2 ATCC) positive control was

inactivated by 0.65-2.061$\log_{10}$ with mean reduction of 0.1.1 $\log_{10}$ and 95% confidence level of ±0.76 $\log_{10}$. While these numbers suggest variability in the group I (MS2 ATCC) positive control, it should be noted that only one of the positive control tests reached a 2.1 $\log_{10}$ reduction and one other isolate inactivated at 1.5 $\log_{10}$. All of the other group I (MS2 ATCC) positive control isolates were inactivated within a relatively small range of 0.40-0.76 $\log_{10}$. The inactivation observed in this particular positive control test may have occurred because of aggregation of the virus particles. Additionally, it is noteworthy that the as a whole, the F+ RNA group I isolates had a relatively narrow range of inactivation from about 0.5 to 2.0 $\log_{10}$. These results suggest generally similar response and relatively high resistance to this temperature.

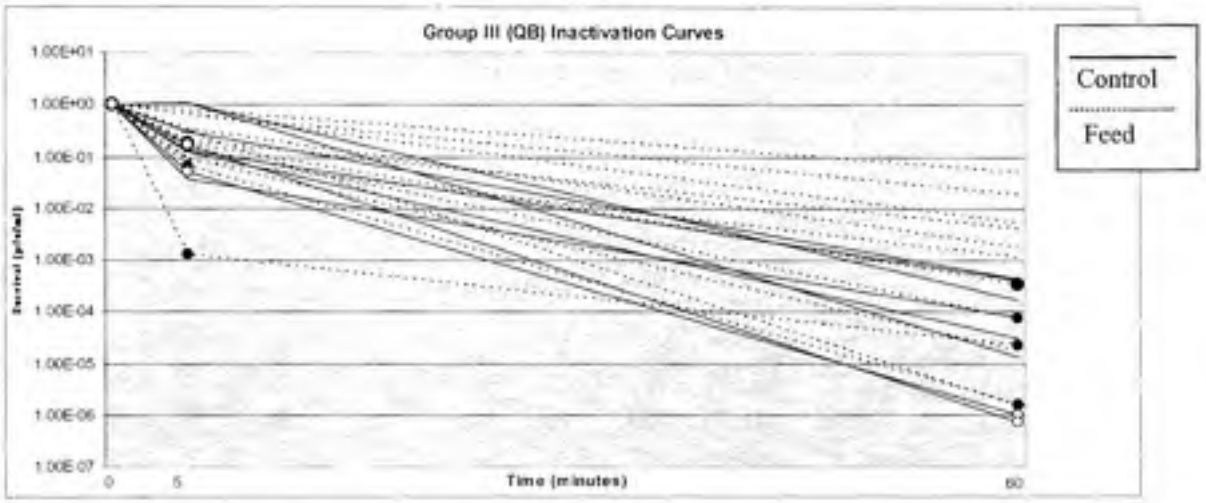


Figure 11. Inactivation Summary Group III (Qβ) Feed and Control Isolates at 53°C for 5 and 60 minutes

As shown in Figure 11, $\log_{10}$ inactivation of the group III F+ coliphage isolates from the feed sludge after 60 minutes at 53 °C ranged from 1.6 to > 5.9 with a mean of 3.8 and 95% confidence level of ±1.5. $\log_{10}$ inactivation of the group III (Qβ) controls after 60

minutes at 53 °C ranged from 3.4 to > 6.1 with a mean of 4.5 and 95% confidence of ±1.1. Plotted lines for isolates shown with the larger black (feed isolates) and white (control isolates) dots indicate samples that had no detectable phage (a concentration below the lower detection limit of the assay) at 60 minutes. For samples with no detectable phages, concentrations were assumed to be 50 pfu/ml for calculation and comparison purposes; however the results displayed for these samples may underestimate the true extent of inactivation of these group III (Qβ) coliphages. The results for the F+ group III coliphages indicate not only greater inactivation of these phages than the group I F+ RNA coliphages, but also a wider range of variability in their response. In general, it appears that the positive control coliphages were more susceptible to thermal inactivation at 53 °C than were the feed sludge isolates. However, some sludge feed isolates also were extensively inactivated.

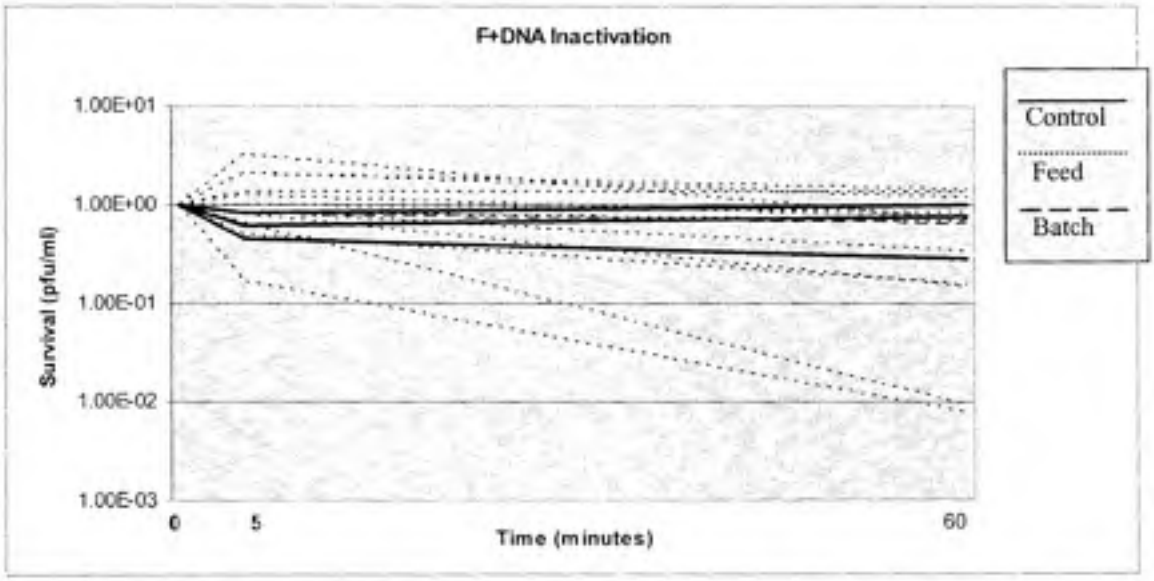


Figure 12. Inactivation Summary F+DNA Coliphage Field and Control Isolates at 53 °C for 5 and 60 minutes

As shown in Figure 12, $\log_{10}$ inactivation of F+ DNA feed sludge isolates was between -0.15-2.1 log with a mean of 0.50 and 95% confidence level of ±0.50 and inactivation of the F+ DNA control isolates was between -0.01 to 0.54 with mean of 0.21 and 95% confidence level of ±0.29. Log_{10} inactivation of the only F+ DNA isolate was 0.18. Figure 12 illustrates apparent increases in the concentration of F+ DNA sludge feed isolates in the first 5 minutes. However, because the actual numbers of detectable phages was low and the confidence intervals for these counts are rather high, these slight increases or decreases in titer at 5 and even 60 minutes can be attributed to statistical variability or chance. However, it is noteworthy that these isolates appear to be inactivated at the same or very similar slow rate and to a similar extent, with less than 1 $\log_{10}$ inactivation after 60 minutes. Although the majority of F+ DNA coliphage isolates are relatively resistant to a temperature of 53 °C for up to 60 minutes of exposure, 2 isolates from the feed sludge were inactivated somewhat more extensively, with about 2 $\log_{10}$ inactivation after 60 minutes at 53 °C. These isolates however were characterized and do not belong to a specific F+ DNA subgroup. One isolate was Fd-like and the other CH-M13-like.

Results Summary

The removal F+ coliphages were evaluated in a lab scale thermophilic anaerobic digester, which included an continuous flow and subsequent batch reactor, at 51 °C, 53 °C, and 55 °C. In these studies the inactivation of F+ coliphages increases with exposure to thermophilic temperatures. Additionally greater than a two-$\log_{10}$ reduction was seen at 55 °C despite the relatively short residence time at this temperature. Subsequent inactivation

experiments in the batch reactor provided kinetic information suggesting that there may be heat-sensitive and heat-resistant populations of coliphages.

Isolates were then collected from feed sludge and at various times during batch treatment and characterized. The predominance of F+ RNA in the batch reactor further indicated that the F+ coliphages may exhibit different heat resistances. Samples of F+ DNA and F+ RNA group I and group III were exposed to a lab scale thermal inactivation experiment at 53 °C, a temperature used in TAD.

After conducting the lab scale thermal inactivation experiments at 53 °C, it was apparent that F+ RNA group III coliphages are significantly more heat-sensitive than the F+ DNA or F+ RNA group I coliphages. This information is useful in interpreting which coliphage group or type most closely resembles human enteric virus inactivation in the presence of thermophilic temperatures and may be able to predict pathogen reduction in a thermophilic digestion process.

V DISCUSSION

Thermophilic Anaerobic Digestion Experiments

A laboratory-scale, continuous-flow thermophilic anaerobic digester was capable of stable operation at a short residence time (four to six days) over a temperature range from 51 °C to 55 °C with influent sludge from three different sources (Aitken et al, submitted).

Male-specific (F+) coliphages were reduced more consistently and extensively than somatic coliphages in the continuous-flow thermophilic digester. F+ coliphages were reduced by 0.86-1.57 $\log_{10}$ at 53 °C and > 2-$\log_{10}$ at 55 °C. Somatic coliphages were reduced by 0.50-1.64 $\log_{10}$ removal with no apparent trend in temperature. (Aitken et al, submitted). The coliphage inactivation results from the continuous flow thermophilic digester suggested an apparent effect of temperature on the extent of inactivation, with increased inactivation at higher thermophilic temperature. However, this apparent effect of increased temperature causing increased inactivation was not supported by the results of additional reduction experiments on the surviving coliphages in the continuous flow reactor effluent when subsequently exposed to thermophilic temperatures in the batch reactor. In these experiments $\log_{10}$ reductions were 1.07-1.35 at 51 °C, > 0.05-1.11 at 53 °C, and > 0.42 at 55 °C, respectively.

Subsequent batch inactivation experiments comparing the inactivation rates at 51 °C and 53 °C (Table 7) also did not indicate that the extent of inactivation was a function of temperature. However, these inactivation experiments did suggest that there was more rapid

initial inactivation of a fraction of the coliphages and then a persistent fraction. About 2 to 3 $\log_{10}$ of the initial coliphages are inactivated relatively rapidly, within 2 hours, and the remaining ones were very persistent, with little further reduction in coliphage concentration for the duration of the 24-hour experiment. It should be noted that in these batch inactivation studies, only a few experiments were conducted and they were conducted on different batches of sludges. The indigenous coliphages and the entire range of thermophilic digestion temperatures were not evaluated for each batch. Therefore the ability to make broad conclusions about increased thermophilic temperatures on coliphage inactivation is very limited.

The overall findings for inactivation of somatic and male-specific and somatic coliphages in this study are consistent with observations made by others that coliphages are more heat-resistant than enteric viruses (Mocé-Llivina et al., 2003). Mocé-Llivina et al. evaluated the inactivation of bacterial indicators and bacteriophages by thermal treatment of dewatered sludge at 80 °C and raw sewage at 60 °C. In both cases the bacteriophages were more resistant to thermal inactivation than the bacterial indicators, except for sulfite-reducing clostridia spores. Of the bacteriophages studied, the F+ phages were more heat sensitive than the somatic and *B. fragilis* coliphages. (Mocé-Llivina et al. 2003).

The combined data for coliphage inactivation from the continuous flow digester and from batch thermophilic digestion treatment of effluent biosolids from the continuous digester suggested differences in coliphage susceptibility to thermophilic anaerobic digestion, with some more susceptible and some more resistant. Therefore, it was of interest to determine if different coliphages in the sludge did in fact differ in their rate and extent of inactivation by thermophilic anaerobic temperature conditions. It was hypothesized that the

effects of temperature probably were the major factor causing coliphage inactivation in the thermophilic anaerobic digestion process and that different coliphages had different susceptibilities to temperatures in the thermophilic range. The results from the thermal batch inactivation rate experiments at 51 °C and 53 °C suggested that there is a fraction of the male-specific coliphages that is more resistant to heat than the remaining phages in this group. This was further documented by showing that different F+ coliphages subgroups differed in the rate and extent to which they are inactivated by thermophilic temperatures. In general group III F+ RNA coliphages were inactivated more rapidly and extensively than either group I F+ RNA coliphages or F+ DNA coliphages.

Male-specific coliphages may be a useful indicator of pathogen inactivation during thermophilic anaerobic digestion over the temperature range we evaluated. The fact that male-specific coliphages were removed by up to 2.6 $\log_{10}$/g TS under thermophilic anaerobic conditions lends support to their potential use as an indicator of thermal inactivation of viruses and other pathogens. However, the observation that there are some male-specific coliphages that persist and survive thermophilic anaerobic digestion creates problems in using them as an indicator of the process. Such a heterogeneous response of these male-specific coliphages to thermophilic anaerobic digestion and the extent to which it exists in different sludges detracts from the use of these coliphages as indicators of thermophilic anaerobic digestion. Furthermore, the reasons for this heterogeneous response were unknown until this study was conducted, and the lack of understanding of this persistence further detracted from the potential usefulness of these coliphages as indicators of the process.

It was therefore of interest to characterize a sample of isolates collected from sludges of the feed and batch reactors in order to gain insight into the thermal resistance properties of F+ coliphages initially present in the sludge and those that survived the batch reactor. This information was also useful in understanding which, if any, F+ coliphage genotypes or serotypes were more persistent or more sensitive to the TAD or thermophilic temperatures.

Isolate Characterization

In the continuous flow reactor, there were nearly equivalent numbers of F+ DNA and F+ RNA isolates found (16 F+ RNA and 20 F+ DNA). Of the 16 F+ RNA isolates found, relatively similar numbers of group I and III isolates were found (9 group I, 6 group III, and 1 mixture of both group I and III). In the batch reactor, however, there was a preponderance of F+ RNA coliphages found (73 F+ RNA and 1 F+ DNA). While there was only one F+ DNA isolate found, it was found after 8 hours of exposure in the batch reactor. The F+ RNA isolates of the batch reactor were predominantly group I (66 group I, 0 group III, and 7 mixture of group I and III).

The isolate characterization illustrated that F+ DNA and F+ RNA group I (MS2) and group III (Qβ) are present in comparable amounts in the original feed sludge. The preponderance of group I F+ RNA coliphages at various times in the batch reactor suggested that this serogroup may possess heat-resistant properties. While only one F+ DNA isolate was found in the batch reactor, it was found after 8 hours of exposure to thermophilic digestion. This suggests that F+ DNA coliphages may have some heat resistant properties as well.

It is uncertain as to why so few F+ DNA coliphages were present in the batch reactor. It is possible that the F+ *E. coli* host used in this study has differential binding and infectivity

efficiency for F+ DNA and F+ RNA coliphages. If these two groups of phages are detected with different efficiency, it could bias the results that attempt to determine their relative abundance in different sludge samples. It is known that the F+ DNA coliphages bind to the top of the pili on their bacterial hosts, such as *E. coli* F_{amp}, and the F+ RNA coliphages bind to the sides of the pili where there is more surface area (Bacteriophages 1975; Lubkowski et al. 1999; Karlsson et al. 2003). This difference in receptor binding location and in the possible abundance of receptor binding sites could influence the results observed for the relative abundance of the two different coliphage groups. While there is not literature that states F_{amp} preferentially binds or supports the replication of F+ RNA coliphages, it is perhaps a possibility and it deserves further investigation.

While little is known about the ecology of F+ DNA coliphages (Furuse 1987), they have been found in a variety of water sources impacted by human and animal fecal waste sources and found in comparable percentages as F+ RNA coliphages in waste sources (Cole et al. 2003). In our study the reverse-line blot hybridization experiment illustrated that no one particular type of F+ DNA coliphage was exclusively present or predominant in the raw sludge. CH-like, M13-like, and Fd-like were all present among the isolates tested. Again, the sample size (n=7) of F+DNA coliphages tested was small and it would be useful to study F+ DNA isolates in sludge further before broad conclusions about relative abundance are made.

There is more literature on the ecology of F+ RNA coliphages. It has been cited that F+ RNA strains isolated from human feces are usually groups II and III, while groups I and IV are usually found in animal feces (Hsu et al 1995; Grabow 2001; Furuse 1987; Havelaar 1986, Scott et al. 2002). This information in the past has been postulated to be useful in fecal

source tracking (Scott et al. 2002). Additionally, differences in the relative percentages of F+ RNA coliphage groups found in various waste and water samples were thought to be useful for source tracking of specific inputs (Cole et al 2003). However, Schaper et al. (2002) suggest that using the distribution of genotypes of F+ RNA bacteriophages for fingerprinting the origin of fecal contamination in natural samples requires previous knowledge of the comparative resistance of the four genotypes to inactivation by various factors.

In this study there was a predominance of group I coliphages surviving thermophilic anaerobic digestion in the continuous-flow and batch reactors. If biosolids from these reactors were to be land applied, the presence of group I isolates in the field may reflect a heat resistance of the thermophilic anaerobic digester rather than a high proportion or load of animal source inputs. This differential survival of F+ RNA coliphage groups may limit the usefulness of F+ RNA coliphage grouping for tracking of fecal sources.

It may be worth noting that, of 66 isolates originally reported as F+ DNA coliphages, only 21 were truly characterized as F+ DNA after confirming with PCR methods. This is consistent with results found by Havelaar et al. (1986), whose survey of RNAse sensitivity yielded relatively many F+ RNA isolates resistant to the RNAse. The serotypes of these isolates were later characterized to be Type IV (FI). With regard to RNAse sensitivity of F+ RNA phages, another series of experiments was conducted (unpublished) and revealed a difference in resistance among serogroups. The order of RNAse sensitivity was II > I >III > IV (Havelaar 1986). Further it has been postulated that free ss-RNA can take on looped hairpin shape where it may mock the double helix nature of the DNA coliphages (Sobsey, personal communication). This property can cause RNA to be resistant to certain types of RNAse.

Based on the results of the biphasic curves with respect to thermal inactivation in the thermophilic anaerobic digester and isolate characterization, it was of interest to additionally test the heat resistance of isolates under controlled temperature exposure conditions. This was done using a thermoregulated heat block at 53 °C, a temperature characteristic of thermophilic digestion processes. A representative sample of the group I and III F+ RNA and F+ DNA isolates were chosen for the experiment. MS2, Qβ, Fd, M13, and F1 lab strain isolates were tested as controls along with the field isolates. They were enumerated before exposure, after 5 minutes and after 60 minutes of exposure. Additionally isolates were titered after being held at room temperature (~25 °C) to insure that the inactivation effects of the coliphages were due to increased heat only in the heatblock model for the exposure temperature in the thermophilic anaerobic digester.

Lab Scale Inactivation Experiments

The thermal analysis of the heatblock exposures indicated that group I F+ RNA coliphages and F+ DNA coliphages are inactivated to a lesser extent than the group III F+ RNA coliphages. There were unexplainable reductions of the same coliphages held at room temperature. This phenomenon was accounted for by subtracting the isolate $\log_{10}$ reductions at room temperature from the $\log_{10}$ inactivation in the heat block and then performing statistical analysis (ANOVA) to examine differences in reductions of different F+ coliphage DNA types and groups.

This experiment illustrated that F+ RNA coliphages, once thought to be a homogeneous group, are actually heterogeneous in their resistance to heat. There was a statistically significant difference in the inactivation of group I (MS2) and group III (Qβ) coliphages after 60 minutes exposure to 53 °C. Our results are consistent with Schaper et al.

(2002), which illustrated comparative data on the susceptibility of bacteriophages of each of the four genotypes of F+ RNA bacteriophages to various inactivating factors.

In studies by Schaper et al. (2002), Brion et al. (2002) and Long and Sobsey (2004), the phage isolates of genotype I survived more successfully compared to phages of other genotypes for various inactivating treatments. In the Schaper et al. study, genotype I was more resistant to all inactivating conditions than bacteriophages of genotypes III and IV and also more resistant than phages of genotype II to most inactivating treatments and conditions. The phage isolates of genotypes III and IV studied were similar in most inactivating conditions studied. It was concluded that the order of resistance to different inactivating factors from greatest to smallest is as follows: genotype I > genotype II > genotype III > genotype IV. Brion et al. (2002) found increased survival of environmentally derived group I and II coliphages at 25 to 37 °C compared to other F+ RNA serotypes.

The reasons for the differences in sensitivities to heat are unknown. Groups I and II belong to the *Leviviridae* family and groups III and IV belong to the *Allolevivirida*e family, meaning these groups do differ genetically. It is possible that these genetic differences form different proteins and/or differences in protein positions or capsid stability. These differences may cause the phage groups to be more or less susceptible to higher temperatures (Vine, Jan personal communication 2004).

Again, not as much is known about F+ DNA coliphage survival in the presence of heat. However, F+ DNA coliphages have been found in the environment in summer months and to be more stable than F+ RNA coliphages over different seasonal temperatures, suggesting they may have some resistance to heat (Chung et al., 1996; Long, Sharon personal communication 2004). In the current study there was not a statistically significant difference

in the inactivation rates between the field strains and lab strains. Additionally, three different types of lab strains were tested (M13, F1, and Fd), and all three of these strains exhibited similar rates of inactivation. According to the scientific literature, the F+ DNA coliphages as a group are more genetically similar than the F+ RNA coliphages (Horiuchi, et al., 1978).

Our results are consistent with Long and Sobsey (2004), which showed that F+ DNA coliphages may have resistance properties. All four lab strains evaluated (M13, fd, f1 and ZJ/2) survived 110 days at 4 °C, but all were inactivated to some extent when incubated at 20 °C for 100 days. Overall there were few differences in survival of F+ DNA coliphages, but the M13 phages were significantly more resistant than the other F+ DNA phages tested. More inactivation studies of field isolates need to be done to identify if the F+ DNA coliphages are truly a more homogeneous group in regards to their heat resistance properties.

There was slight variability in the inactivation rates between the lab strains and field strains within the F+ RNA groups I, group III, and F+ DNA isolates. However, no particular group of field strain was significantly ($P > 0.05$) different than its counterpart lab control strain.

An unexpected die-off occurred in some F+ RNA coliphages at room temperature. While these phages were to be used as controls, several exhibited inactivation close to 2 $\log_{10}$ after 60 minutes at room temperature (~25 °C). The F+ DNA and group III ($Q\beta$) coliphages remained stable at 25 °C. The group I (MS2) however showed greater loss of infectivity. There was a significance difference between the group I batch isolates and the F+ DNA (feed and control) isolates and between the group I batch isolates and the group III ($Q\beta$) control isolates.

It is possible the apparent inactivation seen in the group I isolates may actually be caused by virus aggregation in the TSB media, whose effects may be exacerbated by the spot plate titer assay used. The results of this assay method can be somewhat difficult to interpret, especially because the plaque sizes are very small. An aggregation of virions may contain many individual virus particles, but only be represented as one plaque forming unit (pfu) to the naked eye. The possibility of viral aggregation is strengthened further by the fact that these isolates also exhibited more retardant inactivation curves ("tailing effect") in the kinetic inactivation graphs, which is thought to be characteristic of virus aggregation (Maier, et al 2000).

The use of another medium, such as beef extract or PBS, may be more efficient in reducing the effects of aggregation. However, a small comparison study was conducted between the double agar layer method and the spot titer method. In this study there was no significant difference between the results of two methods. Additionally in the same study a dilution media comparison test was conducted using 3% Beef Extract, phosphate buffer solution (PBS), PBS (Dubeccos), and TSB. The dilution media did not appear to adversely affect the counts for the spot titer procedure.

The data on infectivity titer versus time for every isolate was plotted to illustrate if the inactivation rate followed first-order kinetics (linear) or a two-population model (tailing effect). All isolate groups almost exclusively followed first-order inactivation kinetics, with the exception of the group I batch isolates. Again, this could possibly be due to aggregation of the group I coliphages in the media, which could be further exacerbated by the spot plate titer assay used.

These results are consistent with those of Adams (1959), who stated that inactivation kinetics of bacteriophages were first-order. This can be helpful in predicting bacteriophage inactivation and therefore possibly enteric virus inactivation in thermophilic digestion processes.

In this study, the heat resistances of F+ DNA and group I and III F+ RNA coliphages were evaluated. The group III F+ RNA coliphages exhibited a greater sensitivity to exposure at 53 °C. However, the heterogeneous responses of the F+ coliphages detract from their usefulness as indicators of pathogen reductions in thermophilic digestion processes. In the thermophilic anaerobic digester the enteric virus used, poliovirus type 1, strain LSc, was inactivated very quickly and was not recovered from the effluent of the continuous flow reactor. Therefore, it is of value to target the coliphage or coliphage group that most closely resembles the inactivation of enteric viruses such as poliovirus instead of all F+ coliphages. If methods to analyze just the more heat-sensitive fraction of male-specific RNA coliphages can be developed, some of the "background noise" in the measurements caused by the presence of heat-resistant phages might be removed. Further efforts to develop methods that selectively detect this fraction should be investigated and additional heat inactivation studies should be conducted that directly compare and simultaneously test coliphage inactivation rates to those of enteric viruses, helminthes, and *Salmonella*.

VI CONCLUSIONS

1. F+ RNA coliphages are potentially useful indicators of pathogen reductions in thermal processes to produce Class A biosolids.

2. More specifically, F+ coliphages may be a useful indicator of pathogen inactivation during TAD over the temperature range studied (51-55 °C).

3. These coliphages are similar in morphology to enteric viruses, can be analyzed by simple, inexpensive and rapid methods, and are present in the raw sewage and primary sludge at levels sufficient to determine if $\log_{10}$ pathogen reduction targets are achieved.

4. Observed male-specific coliphage reductions by up to 2.6 $\log_{10}$/g TS under thermophilic anaerobic conditions lends support to their potential use as an indicator of thermal inactivation of sludge pathogens.

5. F+ coliphages gave a heterogeneous inactivation response to thermophilic digestion and to thermal inactivation by its temperatures. This heterogeneity was due to different F+ coliphages, with group III F+ RNA coliphages inactivated more rapidly than group I F+ RNA coliphages of F+ DNA coliphages. This nucleic acid type and group-specific F+ coliphage difference to TAD and the extent to which it exists in different sludges detracts from the usefulness of these coliphages as indicators of pathogen reductions by TAD.

6. F+ RNA group III coliphages appear to have inactivation kinetics predictive of the inactivation of human enteric viruses and possibly other pathogens in sludges subjected to thermal and thermophilic processes.

7. Because of the differences in heat resistances among the F+ RNA coliphages it is postulated that group III coliphages might more accurately follow the inactivation rates of enteric viruses, such as poliovirus.

8. Further study is needed on the heat resistance properties of the F+ RNA and F+ DNA coliphages, as well as, comparisons heat inactivation kinetics between the coliphage types and the various enteric pathogens, such as enteric viruses, helminthes, and *Salmonella* bacteria.

9. Work should be conducted to create an assay that will effectively detect only group III F+ RNA coliphages, the coliphage group that most reliably represents pathogen inactivation in thermophilic anaerobic digestion processes.

APPENDICIES

APPENDIX 1. Continuous Flow Male-Specific Coliphage Data

Continuous Flow Male-Specific Coliphage Data				
SCWRF 55 °C A	**Feed 1**	**Feed 2**	**Effluent**	**Log 10 Reduction**
Run 1 (9/8/2002)	4.06	no data	2.03	
Run 2 (9/15/02)	4.74	4.96	2.43	
Mean	**4.59**		**2.23**	**2.36**
Std Dev	**0.47**		**0.28**	**0.55**
SCWRF 55 °C B	**Feed 1**	**Feed 2**	**Effluent**	
Run 1 (12/14/03)	4.44	-	1.84	
Mean	**4.44**		**1.84**	**2.60**
Std Dev	-		-	-
SCWRF-53 °C	**Feed 1**	**Feed 2**	**Time 0**	
Batch Run 1 (1/18/03)	3.02	3.76	2.18	
Batch Run 2 (1/26/03)	3.76	NA	2.20	
Run 1 (2/1/03)	3.05		2.20	
Run 2 (2/8/03)	2.95		2.11	
Mean	**3.308**		**2.173**	**1.14**
Std dev.	**0.414**		**0.043**	**0.42**
SCWRF-51 °C	**Feed 1**	**Feed 2**	**Effluent**	
Run 2 (3/8/03)	2.87			
Run 1 (3/15/03)		3.77	3.89	
Run 2 (3/23/03)	3.54	3.28	2.33	
Mean	**3.37**		**3.11**	**0.25**
Std. Dev	**0.39**		**1.10**	**1.17**
OWASA-53 °C	**Feed 1**	**Feed 2**	**Effluent**	
Run 1 (4/5/03)	1.72	1.48	1.14	
Run 2 (4/12/03)	0.20	1.42	-0.44	
Mean	**1.21**		**0.35**	**0.86**
Std Dev	**0.68**		**1.12**	**1.31**
WLSSD-53 °C	**Feed 1**	**Feed 2**	**Effluent**	
Run 1 (4/26/03)	2.12	2.11	0.61	
Run 2 (5/3/03)	2.32	2.32	0.69	
Mean	**2.22**		**0.65**	**1.57**
Std Dev	**0.12**		**0.06**	**0.13**

APPENDIX 2. Log_{10} Concentration (MPN/L) of Male-Specific Coliphages in Batch Reactor Inactivation Experiments

Time, hr	Log_{10} Concentration, MPN/L			
	51 °C (1)	51 °C (2)	53 °C (1)	53 °C (2)
0	4.954	4.699	4.380	3.903
0.5	4.041	3.699	3.278	3.699
1	3.477	3.322	2.652	2.262
2	2.954	3.114	2.235	2.500
4	3.362	2.602	1.831	2.121
8	2.301	2.903	2.262	2.121
24	2.301	2.602	1.522	1.831

APPENDIX 3. Isolate Characterization in Feed and Batch Reactors

Isolate Totals			
Feed		**Batch**	
F+DNA	20	F+DNA	1
F+RNA	16	F+RNA	73
MS2	9	MS2	66
Qβ	6	Qβ	0
m/q	1	m/q	7
un-id	1	un-id	10

Feed Run	Feed Number	A	B	C	D	E
Spiked Batch Test-51 °C –Run 2	Feed	DNA	DNA	DNA		
Continuous Batch Test-51 °C –Run 2	Feed 1	MS2	Qβ	Qβ	Qβ	MS2
	Feed 2	MS2	MS2	DNA	Qβ	DNA
OWASA-53 °C Run 1	Feed 1	DNA	DNA			
	Feed 2	Qβ	DNA			Qβ
OWASA-53 °C Run2	Feed 1	DNA	DNA			
	Feed 2	DNA	DNA	un-id		
Deluth-53 °C Run 1	Feed 1	MS2		MS2		
	Feed 2	DNA	DNA	DNA		
Deluth-53 °C Run2	Feed 1	DNA	DNA	DNA	MS2	
	Feed 2	MS2	DNA	DNA	m/q	

* m/q indicates presence of both type 1 (MS2) and type III (Qβ) phage present

** All isolates are field isolates. Therefore MS2 = Type I F+RNA phage and Qβ = Type III F+RNA phage

Batch Run	Time Sampled	A	B	C	D	E
Spiked Batch Test-51 °C –Run 1	Time 0	MS2	MS2	MS2		
	Time 0.5	MS2	MS2	MS2		
	Time 1	MS2	MS2			
	Time 2	MS2	MS2	MS2		
	Time 4	MS2	MS2			
	Time 8	DNA	MS2	MS2		
	Time 24	MS2	MS2			
Spiked Batch Test-51 °C –Run 2	Time 0	m/q	m/q	m/q		
	Time 0.5	m/q	m/q	m/q		
	Time 1	m/q	MS2	MS2		
	Time 2	MS2	MS2	MS2		
	Time 4	MS2	MS2	MS2		
	Time 8	MS2	MS2	MS2		
	Time 24	MS2	MS2			
Continuous Batch Test-51 °C –Run 2	Time 0	MS2	MS2	MS2	MS2	MS2
	Time 1	MS2	MS2	MS2	MS2	MS2
	Time 3	MS2	MS2	MS2		
	Time 9	MS2	MS2	MS2		
	Time 24	MS2	MS2	MS2		
OWASA-53 °C Run 1	Time 0	MS2				
	Time 3	MS2				
Deluth-53 °C Run 1	Time 0	MS2	MS2	MS2	MS2	
	Time 1	MS2	MS2	MS2	un-id	
	Time 3		MS2	MS2	MS2	
	Time 9	MS2				
	Time 24	MS2				
Deluth-53 °C Run 2	Time 0	MS2	MS2		un-id	
	Time 1	un-id	un-id	MS2		
	Time 3		un-id	un-id		
	Time 9	un-id	un-id	un-id		
	Time 24	un-id				

* m/q indicates presence of both type 1 (MS2) and type III ($Q\beta$) phage present

APPENDIX 4. Raw Data for Lab-Scale Heat Inactivation Experiments at 53 °C

F = Entire zone of lysis (not spotty) observed
T = Too numerous to count, but zone of lysis is spotty

Group I Feed (1a)								
dilution	initial		5 minutes		60 minutes		Final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	F	F
0.00002	F	F	F	F	T	T	T	T
0.000002	T	T	T	T	40	26	50	66
0.0000002	26	23	33	36	5	3	9	13
TOTAL	245000000		345000000		33636363.64		62727272.73	

Group I Feed (1b)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	F	F
0.00002	F	F	F	F	T	T	T	T
0.000002	T	T	T	T	5	11	35	40
0.0000002	16	19	19	13	0	0	9	2
TOTAL	175000000		160000000		8000000		39090909.09	

Group I Feed (2a)								
dilution	Initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	F	F
0.00002	F	F	F	F	90	53	T	T
0.000002	41	34	35	38	21	5	21	24
0.0000002	8	5	5	4	1	1	1	4
TOTAL	40000000		37272727.27		7702702.703		22727272.73	

Group I Feed (2b)								
dilution	Initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	F	F
0.00002	F	F	F	F	45	53	T	T
0.000002	34	38	44	44	1	1	15	15
0.0000002	8	5	2	6	1	0	1	1
TOTAL	38636363.64		43636363.64		4549549.55		14545454.55	

Group I Feed (3)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	T	T	F	F
0.002	F	F	F	F	T	T	F	F
0.0002	F	F	F	F	43	44	T	T
0.00002	T	T	T	T	8	5	T	T
0.000002	50	55	27	42	2	0	7	15
0.0000002	4	3	3	5	0	0	1	0
0.00000002	1	1	0	0	0	0	0	0
TOTAL	51351351.35		35000000		459459.4595		10454545.45	

Group I Feed (4)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	T	T	T	T
0.0002	F	F	T	T	T	T	T	T
0.00002	T	T	T	T	13	12	32	28
0.000002	8	16	9	9	1	1	2	3
0.0000002	2	0	0	0	0	0	0	0
0.00000002	0	0	0	0	0	0	0	0
TOTAL	11818181.82		9000000		1227272.727		2954545.455	

Group I Feed (5)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	F	F
0.00002	F	F	T	T	17	25	T	T
0.000002	T	T	T	T	2	2	13	23
0.0000002	23	27	9	9	0	0	0	2
0,00000002	3	4	2	0	0	0	0	0
TOTAL	259090909.1		90909090.91		2090909.091		17272727.27	

Group I Feed (6)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	T	T	F	F
0.0002	F	F	F	F	T	T	T	T
0.00002	T	T	T	T	6	7	T	T
0.000002	23	28	17	15	1	0	5	8
0.0000002	2	4	2	2	0	0	0	2
0.00000002	1	0	0	0	0	0	0	0
TOTAL	26126126.13		16363636.36		636363.6364		6818181.818	

Group I Feed (7)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	T	F	T	T	T	T
0.00002	F	F	T	T	35	8	T	T
0.000002	T	T	T	T	5	1	6	5
0.0000002	19	26	17	30	0	0	0	0
0.00000002	1	1	1	1	0	0	0	0
TOTAL	213636363.6		222727272.7		2227272.727		5500000	

Group I Feed (8)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	T	T	F	F
0.0002	F	F	F	F	T	T	T	T
0.00002	T	T	T	T	4	7	3	30
0.000002	38	55	15	21	0	0	0	1
0.0000002	5	5	2	1	0	0	0	0
0.00000002	0	0	3	0	0	0	0	0
TOTAL	46818181.82		18918918.92		550000		1545454.545	

Group I Batch (1a)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	T	T
0.00002	T	T	T	T	T	T	T	T
0.000002	24	27	30	23	6	0	5	2
0.0000002	2	0	1	4	0	0	0	0
0.00000002	0	0	0	0	0	0	0	0
TOTAL	240909909.09		26363636.36		3000000		3500000	

Group I Batch (1b)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	T	T	T	T
0.000002	T	T	T	T	25	25	54	42
0.0000002	17	22	10	9	1	4	4	3
0.00000002	2	1	3	1	0	0	0	0
TOTAL	190909090.9		104545454.5		25000000		46818181.82	

Group I Batch (3a)																
dilution	initial				5 minutes				60 minutes				final			
0.02	F	F			F	F			F	F			F	F		
0.002	F	F			F	F			F	F			F	F		
0.0002	F	F			F	F			T	T			F	F		
0.00002	F	F			F	F			T	T			T	T		
0.000002	T	T			T	T			7		8		16		16	
0.0000002		36		30		10		23		2		3		1		0
0.00000002		8		5		4		0		0		0		0		0
TOTAL		359090909.1				168181818.2				9090909.091				15000000		

Group I Batch (3b)																
dilution	initial				5 minutes				60 minutes				final			
0.02	F	F			F	F			F	F			F	F		
0.002	F	F			F	F			F	F			F	F		
0.0002	F	F			T	T			T	T			T	T		
0.00002	T	T				36		42		10		10		12		24
0.000002		20		28		0		4		0		0		0		2
0.0000002		1		1		0		1		0		0		0		0
0.00000002		0		0		0		0		0		0		0		0
TOTAL		22727272.73				3738738.739				1000000				1727272.727		

Group I Batch (4)																
dilution	initial				5 minutes				60 minutes				final			
0.02	F	F			F	F			F	F			F	F		
0.002	F	F			F	F			T	T			F	F		
0.0002	F	F			T	T			5		2	T		T		
0.00002	T	T				13		20		1		0		3		3
0.000002		16		15		2		4		0		0		0		0
0.0000002		0		2		0		0		0		0		0		0
0.00000002		0		0		0		0		0		0		0		0
TOTAL		15000000				1772727.273				36363.63636				300000		

Group I Batch (5)																
dilution	initial				5 minutes				60 minutes				final			
0.02	F	F			F	F			F	F			F	F		
0.002	F	F			F	F			F	F			F	F		
0.0002	F	F			F	F			T	T			T	T		
0.00002	T	T			T	T			13		11		18		29	
0.000002		39		31		16		14		1		3		0		0
0.0000002		3		8		1		2		1		0		0		0
0.00000002		0		0		0		1		0		0		0		0
TOTAL		36818181.82				15315315.32				1306306.306				2350000		

Group I Batch (6)										
dilution	initial			5 minutes			60 minutes		final	
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	T	T	T	T		
0.0002	T	T	T	T	12	8	T	T		
0.00002	37	19	10	8	0	0	7	2		
0.000002	1	1	3	1	0	0	0	1		
0.0000002	0	0	0	0	0	0	0	0		
0.00000002	0	0	0	0	0	0	0	0		
TOTAL	2636363.636		1000000		100000		454545.4545			

Group I Batch (7)										
dilution	initial			5 minutes			60 minutes		final	
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	T	T	F	F		
0.0002	F	F	F	F	T	T	F	F		
0.00002	T	T	T	T	29	45	T	T		
0.000002	9	7	10	9	1	2	9	8		
0.0000002	2	1	0	2	0	0	0	0		
0.00000002	0	0	0	0	0	0	0	0		
TOTAL	8636363.636		9545454.545		3500000		8500000			

Group I Batch (8)										
dilution	initial			5 minutes			60 minutes		final	
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	T	T	T	T		
0.0002	F	F	F	F	T	T	T	T		
0.00002	F	F	T	T	5	12	T	T		
0.000002	T	T	T	T	0	2	11	4		
0.0000002	4	4	5	7	1	0	0	0		
0.00000002	1	0	1	0	0	0	0	0		
TOTAL	40909090.91		59090909.09		900900.9009		7500000			

Group I Batch (9)										
dilution	initial			5 minutes			60 minutes		final	
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	T	T	F	F		
0.0002	F	F	F	F	T	T	T	T		
0.00002	F	F	T	T	15	1	T	T		
0.000002	T	T	49	40	0	0	8	10		
0.0000002	19	8	0	6	0	0	0	0		
0.00000002	1	3	0	0	0	0	0	0		
TOTAL	140909090.9		43181818.18		800000		9000000			

Group I Batch (10)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	T	F	F	F
0.0002	F	F	F	F	T	T	F	F
0.00002	F	F	T	T	1	20	T	T
0.000002	52	46	14	13	0	0	13	4
0.0000002	3	8	1	0	0	0	1	3
0.00000002	1	0	1	0	0	0	0	0
TOTAL	49549549.55		13063063.06		1050000		9545454.545	

Group I Batch (11)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	T	F	F	F	F
0.0002	F	F	F	F	T	T	T	T
0.00002	F	F	T	T	T	T	F	F
0.000002	T	T	48	52	4	2	T	T
0.0000002	37	30	5	4	0	0	3	4
0.00000002	3	0	0	0	0	0	0	1
TOTAL	318181818.2		49545454.55		3000000		36363636.36	

Group I Batch (12)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	T	T
0.00002	F	F	T	T	12	3	T	T
0.000002	T	T	26	12	3	0	5	4
0.0000002	8	5	3	1	0	0	0	0
0.00000002	1	1	0	0	0	0	1	0
TOTAL	68181818.18		19090909.09		818181.8182		4950495.05	

Group I Batch (13)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	T	T	F	F
0.0002	F	F	T	T	T	T	T	T
0.00002	T	T	27	30	9	3	17	15
0.000002	21	10	4	3	0	0	5	1
0.0000002	3	2	0	0	0	0	0	0
0.00000002	1	1	0	0	0	0	0	0
TOTAL	17117117.12		2909090.909		600000		1727272.727	

MS2 Control (1)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	F	F	T	T
0.000002	T	T	T	T	T	T	T	T
0.0000002	40	29	21	35	0	13	18	23
0.00000002	3	7	3	2	0	0	3	2
0.000000002	0	1	0	0	0	0	1	0
TOTAL	360360360.4		277272727.3		65000000		211711711.7	

MS2 Control (2)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	T	T	F	F
0.000002	F	F	F	F	10	13	T	T
0.0000002	T	T	T	T	0	2	T	T
0.00000002	8	18	12	12	0	0	4	10
TOTAL	1300000000		1200000000		11363636.36		700000000	

MS2 Control (3)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	F	F	F	F
0.000002	F	F	T	T	T	T	T	T
0.0000002	T	T	53	69	20	27	30	15
0.00000002	13	12	5	8	1	0	0	0
TOTAL	1250000000		613636363.6		218181818.2		225000000	

MS2 Control (4)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	F	F	F	F
0.000002	F	F	T	T	T	T	T	T
0.0000002	T	T	T	T	T	T	T	T
0.00000002	11	16	10	9	3	3	3	8
TOTAL	1350000000		950000000		300000000		550000000	

MS2 Control (5)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	F	F	F	F
0.000002	F	F	T	T	T	T	T	T
0.0000002	45	30	T	T	14	12	19	18
0.00000002	8	11	3	8	2	5	4	6
TOTAL	427272727.3		550000000		150000000		213636363.6	

Group III Feed (1)								
dilution	initial		5 minutes		60 minutes		Final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	38	34	F	F
0.0002	F	F	F	F	9	4	F	F
0.00002	T	T	40	24	0	0	T	T
0.000002	T	T	0	0	0	0	18	23
0.0000002	5	3	0	0	0	0	2	4
TOTAL	40000000		3200000		38636.36364		21363636.36	

Group III Feed (2a)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	T	F
0.00002	T	T	F	F	10	10	T	T
0.000002	T	T	22	34	0	0	32	30
0.0000002	4	7	2	5	0	0	2	4
0.00000002	0	0	0	0	0	0	0	0
0.000000002	0	0	0	0	0	0	0	0
TOTAL	55000000		28636363.64		1000000		30909090.91	

Group III Feed (2b)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	T	T	F	F
0.00002	F	F	F	F	T	T	F	F
0.000002	T	T	T	T	11	3	T	T
0.0000002	24	19	15	17	3	0	10	7
0.00000002	1	3	2	1	0	0	0	0
TOTAL	213636363.6		159090909.1		7727272.727		85000000	

Group III Feed (3)										
dilution	initial		5 minutes		60 minutes		final			
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	T	T	F	F		
0.0002	F	F	F	F	T	T	F	F		
0.00002	F	F	T	T	6	8	T	T		
0.000002	T	T	40	45	2	0	11	3		
0.0000002	T	T	7	5	0	0	0	0		
0.00000002	2	2	1	0	0	0	0	0		
TOTAL	200000000		44144144.14		727272.7273		7000000			

Group III Feed (4)										
dilution	initial		5 minutes		60 minutes		final			
0.02	F	F	33	37	0	0	F	F		
0.002	F	F	5	2	0	0	T	T		
0.0002	T	T	1	0	0	0	9	10		
0.00002	27	17	0	0	0	0	0	4		
0.000002	5	0	0	0	0	0	0	1		
0.0000002	0	0	0	0	0	0	0	0		
0.00000002	0	0	0	0	0	0	0	0		
TOTAL	2227272.727		3513.513514		0		108108.1081			

Group III Feed (5)										
dilution	initial		5 minutes		60 minutes		final			
0.02	F	F	F	F	0	0	F	F		
0.002	F	F	F	F	0	2	F	F		
0.0002	T	T	T	T	0	0	F	F		
0.00002	T	T	T	T	0	0	T	T		
0.000002	T	T	8	5	0	0	5	10		
0.0000002	7	6	1	0	0	0	0	1		
0.00000002	0	0	0	0	0	0	0	0		
TOTAL	65000000		6363636.364		1000		7272727.273			

Group III Feed (6)										
dilution	initial		5 minutes		60 minutes		final			
0.02	F	F	F	F	0	0	F	F		
0.002	F	F	T	F	0	0	F	F		
0.0002	F	F	T	T	0	0	T	T		
0.00002	T	T	T	T	0	0	T	T		
0.000002	T	T	10	10	0	0	2	3		
0.0000002	2	6	0	0	0	0	0	0		
0.00000002	1	0	0	0	0	0	0	0		
TOTAL	40909090.91		10000000		0		2500000			

Group III Feed (7)										
dilution	initial			5 minutes		60 minutes			final	
0.02	F	F		F	F	0	0	F	F	
0.002	F	F		F	F	0	0	F	F	
0.0002	F	F		T	T	0	0	T	T	
0.00002	T	T		19	20	0	0	T	T	
0.000002	T	T		3	3	0	0		1	4
0.0000002	4		3	0	0	0	0		0	0
0.00000002	0		0	0	0	0	0		0	0
TOTAL	35000000			2045454.545		0			2500000	

Group III Feed (8)										
dilution	initial			5 minutes		60 minutes			final	
0.02	F	F		F	F		F	F	F	
0.002	F	F		F	F	T	T	F	F	
0.0002	F	F		F	F	1	20	F	F	
0.00002	T	T		T	T	0	1	T	T	
0.000002	T	T		T	T	0	0		10	11
0.0000002	2		7	2	1	0	0		0	2
0.00000002	0		0	0	0	0	0		0	0
TOTAL	45000000			15000000		100000			10454545.45	

Group III Feed (9)										
dilution	initial			5 minutes		60 minutes			final	
0.02	F	F		F	F	0	0	F	F	
0.002	F	F		T	T	0	0	F	F	
0.0002	T	T		8	13	0	0	T	T	
0.00002	7		7	2	0	0	0		3	3
0.000002	0		0	0	0	0	0		0	0
0.0000002	0		0	0	0	0	0		0	0
0.00000002	0		0	0	0	0	0		0	0
TOTAL	700000			104545.4545		0			300000	

Group III Feed (10)										
dilution	initial			5 minutes		60 minutes			final	
0.02	F	F		F	F	0	0	F	F	
0.002	T	T		T	T	0	0	F	F	
0.0002	18		17	3	3	0	0		1	8
0.00002	1		4	0	0	0	0		0	0
0.000002	0		0	0	0	0	0		0	0
0.0000002	0		0	0	0	0	0		0	0
0.00000002	0		0	0	0	0	0		0	0
TOTAL	181818.1818			30000		0			45000	

Group III Control (1)										
dilution	initial			5 minutes			60 minutes			final	
0.02	F	F		F	F		F	F	F	F	
0.002	F	F		F	F		20	18	F	F	
0.0002	F	F		F	F		4	4	F	F	
0.00002	T	T		100	100		0	0	F	F	
0.000002	T	T		0	0		0	0	T	T	
0.0000002	19		35	0	0		0	0		4	4
TOTAL	270000000			10000000			20909.09091			40000000	

Group III Control (2)										
dilution	initial			5 minutes			60 minutes			final	
0.02	F	F		F	F		11	7	F	F	
0.002	F	F		F	F		1	3	F	F	
0.0002	F	F		F	F		0	0	F	F	
0.00002	T	T		T	T		0	0	T	T	
0.000002	7		5	5		8	0	0	T	T	
0.0000002	1		0	1		0	0	0		5	7
2E-08	0		0	0		0	0	0		1	0
2E-09	0		0	0		0	0	0		0	0
TOTAL	5909090.909			6363636.364			1000			59090909.09	

Group III Control (3)										
dilution	initial			5 minutes			60 minutes			final	
0.02	F	F		F	F		21	19	F	F	
0.002	F	F		F	F		1	3	F	F	
0.0002	F	F		F	F		0	0	F	F	
0.00002	F	F		F	F		0	0	F	F	
0.000002	T	T		T	T		0	0	T	T	
0.0000002	8		22	10		23	0	0		8	2
2E-08	3		1	4		0	0	0		0	0
TOTAL	154545454.5			168181818.2			2000			50000000	

Group III Control (4)										
dilution	initial			5 minutes			60 minutes			final	
0.02	F	F		F	F		T	T	F	F	
0.002	F	F		F	F		T	T	F	F	
0.0002	F	F		F	F		5	12	F	F	
0.00002	F	F		T	T		0	0	F	F	
0.000002	T	T		55	55		0	0	T	T	
0.0000002	17		24	3		17	0	0		11	11
2E-08	0		2	1		1	0	0		1	0
TOTAL	195454545.5			59459459.46			85000			104545454.5	

Group III Control (5)										
dilution	initial			5 minutes			60 minutes			final	
0.02	F	F		F	F		0	0	F	F	
0.002	F	F		F	F		0	0	F	F	
0.0002	F	F		T	T		0	0	F	F	
0.00002	T	T		21	29		0	0	T	T	
0.000002	T	T		3	3		0	0		29	32
0.0000002	7		2	0	1		0	0		4	5
2E-08	1		1	0	0		0	0		1	0
TOTAL	50000000			2567567.568			0			31981981.98	

Group III Control (6)										
dilution	initial			5 minutes			60 minutes			final	
0.02	F	F		F	F		50	5	F	F	
0.002	F	F		F	F		0	0	F	F	
0.0002	F	F		F	F		0	0	F	F	
0.00002	F	F		T	T		0	0	F	F	
0.000002	T	T		7	13		0	0	T	T	
0.0000002	3		15	3	0		0	0		5	4
2E-08	1		1	2	0		0	0		0	0
TOTAL	90909090.91			11261261.26			2750			45000000	

Group III Control (7)										
dilution	initial			5 minutes			60 minutes			final	
0.02	F	F		F	F		T	T	F	F	
0.002	F	F		F	F		20	23	F	F	
0.0002	F	F		T	T		9	2	F	F	
0.00002	T	T		T	T		0	0	T	T	
0.000002	T	T		8	9		0	0	T	T	
0.0000002	5		7	1	0		0	0		8	4
2E-08	0		0	0	0		0	0		0	0
TOTAL	60000000			8181818.182			24545.45455			60000000	

Group III Control (8)										
dilution	initial			5 minutes			60 minutes			final	
0.02	F	F		F	F		0	0	F	F	
0.002	F	F		F	F		0	0	F	F	
0.0002	F	F		F	F		0	0	F	F	
0.00002	T	T		T	T		0	0	T	T	
0.000002	T	T		7	17		0	0		12	19
0.0000002	7		7	0	0		0	0		0	3
2E-08	1		0	0	0		0	0		0	0
TOTAL	68181818.18			12000000			0			15454545.45	

F+DNA Field Isolates (1)										
dilution	initial		5 minutes		60 minutes		final			
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	T	T	F	F		
0.0002	F	F	T	F	40	35	T	T		
0.00002	T	T	T	T	5	10	11	9		
0.000002	T	T	T	T	0	0	1	0		
0.0000002	4	4	1	5	0	0	0	0		
0.00000002	1	1	0	0	0	0	0	0		
TOTAL	45454545.45		30000000		409090.909		954545.4545			

F+DNA Field Isolates (2)										
dilution	initial		5 minutes		60 minutes		final			
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	F	F	F	F		
0.0002	F	F	F	F	F	F	F	F		
0.00002	T	T	T	T	T	T	T	T		
0.000002	40	40	34	30	26	26	40	60		
0.0000002	2	4	4	4	3	3	4	2		
0.00000002	0	0	1	0	0	0	0	0		
TOTAL	39090909.09		32882882.88		26363636.4		48181818.18			

F+DNA Field Isolates (3)										
dilution	initial		5 minutes		60 minutes		final			
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	F	F	F	F		
0.0002	F	F	F	F	F	F	F	F		
0.00002	F	F	F	F	F	F	F	F		
0.000002	T	T	T	T	T	T	T	T		
0.0000002	24	24	21	24	24	19	22	25		
0.00000002	2	2	1	5	2	0	1	0		
TOTAL	236363636.4		231818181.8		204545455		218181818.2			

F+DNA Field Isolates (4)										
dilution	initial		5 minutes		60 minutes		final			
0.02	F	F	F	F	F	F	F	F		
0.002	F	F	F	F	F	F	F	F		
0.0002	F	F	F	F	F	F	F	F		
0.00002	F	F	F	F	F	F	F	F		
0.000002	F	F	F	F	F	F	F	F		
0.0000002	T	T	T	T	37	31	T	T		
0.00000002	3	6	4	7	3	1	7	8		
TOTAL	450000000		550000000		327272727		750000000			

F+DNA Field Isolates (5)													
dilution	initial			5 minutes			60 minutes			final			
0.02	F	F		F	F		F	F		F	F		
0.002	F	F		F	F		F	F		F	F		
0.0002	F	F		F	F		F	F		F	F		
0.00002	F	F		F	F		F	F		F	F		
0.000002	T	T		T	T		F	F		T	T		
0.0000002	19		16	14		13	10		13	12		7	
0.00000002	1		2	3		1	1		1	0		0	
TOTAL	172727272.7			140909090.9			113636364			95000000			

F+DNA Field Isolates (6)													
dilution	initial			5 minutes			60 minutes			final			
0.02	F	F		F	F		F	F		F	F		
0.002	F	F		F	F		F	F		F	F		
0.0002	F	F		F	F		F	F		F	F		
0.00002	F	F		F	F		F	F		F	F		
0.000002	F	F		F	F		F	F		F	F		
0.0000002	T	T		T	T		T	T		T	T		
0.00000002	15		9	20		12	22		11	11		8	
TOTAL	1200000000			1600000000			1650000000			950000000			

F+DNA Field Isolates (7)													
dilution	initial			5 minutes			60 minutes			final			
0.02	F	F		F	F		F	F		F	F		
0.002	F	F		F	F		F	F		F	F		
0.0002	F	F		F	F		F	F		F	F		
0.00002	F	F		F	F		F	F		F	F		
0.000002	F	F		F	F		F	F		F	F		
0.0000002	T	T		T	T		T	T		T	T		
0.00000002	7		8	17		15	10		11	5		1	
TOTAL	750000000			1600000000			1050000000			300000000			

F+DNA Field Isolates (8)													
dilution	initial			5 minutes			60 minutes			final			
0.02	F	F		F	F		F	F		F	F		
0.002	F	F		F	F		F	F		F	F		
0.0002	F	F		F	F		T	T		F	F		
0.00002	T	T		T	T		28		26	28		35	
0.000002	10		17	12		5	0		4	1		4	
0.0000002	3		6	1		1	0		0	0		0	
0.00000002	0		2	0		0	0		0	0		0	
TOTAL	17117117.12			8636363.636			2636363.64			3090909.091			

F+DNA Field Isolates (9)										
dilution	initial			5 minutes			60 minutes		final	
0.02	F	F		F	F		T	T	F	F
0.002	F	F		F	F		T	T	F	F
0.0002	F	F		T	T		20	6	T	T
0.00002	T	T		T	T		1	0	T	T
0.000002	13		21	4		0	0	0	17	4
0.0000002	1		0	0		2	0	0	0	0
0.00000002	0		0	0		0	0	0	0	0
TOTAL	15909090.91			2727272.727			122727.273		10500000	

F+DNA Field Isolates (10)										
dilution	initial			5 minutes			60 minutes		final	
0.02	F	F		F	F		F	F	F	F
0.002	F	F		F	F		F	F	F	F
0.0002	F	F		F	F		T	T	F	F
0.00002	F	F		T	T		T	T	T	T
0.000002	T	T		T	T		12	2	17	25
0.0000002	3		6	2		6	2	0	0	0
0.00000002	1		1	1		0	0	0	0	0
TOTAL	50000000			40909090.91			7272727.27		21000000	

F+DNA Field Isolates (11)										
dilution	initial			5 minutes			60 minutes		final	
0.02	F	F		F	F		F	F	F	F
0.002	F	F		F	F		F	F	F	F
0.0002	F	F		F	F		F	F	F	F
0.00002	F	F		F	F		F	F	F	F
0.000002	F	F		F	F		F	F	F	F
0.0000002	T	T		T	T		T	T	T	T
0.00000002	13		8	56		12	8	7	5	3
TOTAL	1050000000			3400000000			750000000		400000000	

F+DNA Field Isolates (12)										
dilution	initial			5 minutes			60 minutes		final	
0.02	F	F		F	F		F	F	F	F
0.002	F	F		F	F		F	F	F	F
0.0002	F	F		F	F		F	F	F	F
0.00002	F	F		F	F		F	F	F	F
0.000002	F	F		F	F		F	F	F	F
0.0000002	T	T		T	T		T	T	T	T
0.00000002	8		3	14		9	8	5	4	1
TOTAL	550000000			1150000000			650000000		250000000	

F+DNA Field Isolates (13)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	F	F	F	F
0.000002	T	T	T	T	T	T	T	T
0.0000002	27	26	25	21	9	9	20	14
0.00000002	3	0	1	0	0	1	0	1
TOTAL	254545454.5		213636363.6		86363636.4		159090909.1	

F+DNA Field Isolates (14)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	F	F	F	F
0.000002	F	F	F	F	F	F	F	F
0.0000002	T	T	T	T	T	T	T	T
0.00000002	7	7	8	6	6	6	5	6
TOTAL	700000000		700000000		600000000		550000000	

F+DNA Control (F1)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	F	F	F	F
0.000002	F	F	T	T	T	T	T	T
0.0000002	14	19	16	8	11	17	8	18
0.00000002	6	2	0	1	0	0	1	1
0.000000002	0	0	0	1	1	0	0	0
TOTAL	186363636.4		117117117.1		143564356.4		127272727.3	

F+DNA Control (M13)								
dilution	initial		5 minutes		60 minutes		final	
0.02	F	F	F	F	F	F	F	F
0.002	F	F	F	F	F	F	F	F
0.0002	F	F	F	F	F	F	F	F
0.00002	F	F	F	F	T	T	F	F
0.000002	T	T	T	T	36	38	T	T
0.0000002	18	5	5	5	5	3	6	11
0.00000002	0	3	0	2	1	0	3	2
0.000000002	0	3	0	1	0	0	0	0
TOTAL	130630630.6		58558558.56		37387387.39		100000000	

F+DNA Control (Fd)												
dilution	initial			5 minutes			60 minutes			final		
0.02	F	F		F	F		F	F		F	F	
0.002	F	F		F	F		F	F		F	F	
0.0002	F	F		F	F		F	F		F	F	
0.00002	F	F		F	F		F	F		F	F	
0.000002	T	T		T	T		T	T		T	T	
0.0000002	29		28	22		21	28		33	38		36
0.00000002	5		1	3		7	1		2	1		6
0.000000002	0		0	0		0	0		0	0		0
TOTAL	286363636.4			240909090.9			290909090.9			368181818.2		

APPENDIX 5. Log_{10} Reductions and Statistics of Each Isolate

Samples Heated to 53 °C for 60 minutes (log_{10} reduction) and (95% CI)							
Isolate #	F+RNA					F+DNA	
	Group I Feed	Group I Batch	MS2 ATCC	Group III Feed	QB ATCC	F+ DNA	F+DNA ATCC
1	1.10 (1.06)	1.34 (0.90)	0.74 (0.35)	2.69 (2.87)	4.11 (4.08)	2.05 (2.52)	0.11 (0.04)
2	0.82 (0.91)	2.02 (1.51)	2.06 (2.05)	1.59 (1.55)	3.77 (3.89)	0.17 (0.09)	0.54 (0.77)
3	2.05 (2.02)	2.15 (1.88)	0.76 (0.90)	2.44 (2.74)	4.89 (4.94)	0.06 (0.03)	-0.01 (0)
4	0.98 (0.99)	2.62 (2.31)	0.65 (0.33)	4.65 (3.80)	3.36 (3.16)	0.14 (0.59)	
5	2.09 (2.05)	1.45 (1.23)	1.50 (0.23)	> 4.81	> 6.00	-0.14 (-0.07)	
6	1.12 (1.06)	1.42 (1.19)		> 5.91	4.52 (4.74)	-0.15 (-0.07)	
7	1.61 (1.30)	0.39 (0.70)		> 5.85	3.39 (3.71)	0.81 (0.90)	
8	1.98 (0.99)	1.66 (1.83)		2.65 (2.85)	> 6.13	2.11 (2.06)	
9	1.93 (1.16)	2.25 (2.10)		> 4.15		0.84 (0.92)	
10		1.67 (1.31)		> 3.56		0.15 (0.07)	
11		2.02 (1.49)				-0.07 (-0.04)	
12		1.92 (1.96)				0.47 (0.23)	
13		1.46 (1.20)				0.07 (0.03)	
14						0.18 (0.09)	
Mean	1.52	1.72076923	1.142	3.83	4.52125	0.500769231	0.213333333
Standard Deviation	0.51376064	0.55118753	0.616538725	1.485358169	1.087098859	0.7693987	0.289194283
Range	1.27	2.23	1.41	>4.32	>2.77	2.26	0.55
Minimum	0.82	0.39	0.65	1.59	3.36	-0.15	-0.01
Maximum	2.09	2.62	2.06	>5.91	>6.13	2.11	0.54
Confidence Level(95.0%)	0.39491165	0.33307928	0.765535525	1.062562034	0.90883674	0.464942973	0.718398925

* The ">" sign indicates there was no detectable coliphage after 60 minutes. For calculation purposes only, it was assumed that the concentration was 50 pfu/ml. However, because fewer that this were detectable, the coliphage inactivated to a greater extent than indicated by the calculations.

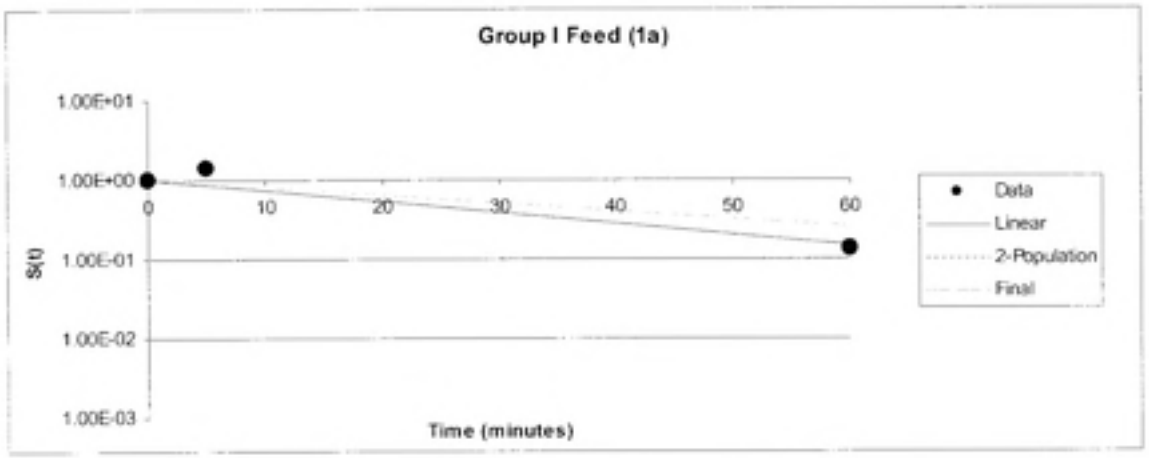
Samples at Room Temperature for 60 minutes (log_{10} reduction) and (95% CI)							
Isolate #	**F+RNA**					**F+DNA**	
	Group I Feed	Group I Batch	MS2 ATCC	Group III Feed	QB ATCC	F+DNA	F+DNA ATCC
1	0.62 (0.83)	1.03 (0.80)	0.23 (0.12)	0.14 (0.33)	0.83 (0.41)	1.68 (1.84)	0.17 (0.08)
2	0.33 (0.17)	1.73 (1.36)	0.27 (0.13)	0.33 (0.41)	**0**	-0.09 (-0.05)	0.12 (0.06)
3	0.69 (0.34)	1.81 (1.72)	0.74 (0.87)	1.46 (1.73)	0.49 (0.22)	0.03 (0.02)	-0.11 (-0.05)
4	0.20 (0.08)	1.70 (1.33)	0.39 (0.19)	1.31 (1.16)	0.27 (0.14)	-0.22 (-0.11)	
5	1.18 (1.09)	1.19 (1.08)	1.34 (0.15)	0.95 (1.00)	0.19 (0.60)	0.10 (0.05)	
6	0.17 (0.60)	0.76 (0.38)		1.21 (1.09)	0.30 ((0.13)	0.40 (0.20)	
7	0.58 (0.29)	0.01 (-0.02)		1.15 (1.07)	0	0.74 (0.87)	
8	1.59 (0.27)	0.74 (0.85)		0.63 (0.84)	0.64 (0.82)	0.18 (0.07)	
9	1.48 (0.96)	1.19 (1.08)		0.37 (0.18)		0.38 (0.67)	
10		0.72 (0.36)		0.61 (0.28)		0.42 (0.21)	
11		0.94 (0.47)				0.34 (0.17)	
12		1.14 (1.05)				0.20 (0.10)	
13		1.00 (1.00)				0.10 (0.05)	
14						0.26 (0.11)	
Mean	0.76	1.073846154	0.594	0.816	0.215	0.327692308	0.06
Standard Deviation	0.534696175	0.490587037	0.462849868	0.460994095	0.556494128	0.476115424	0.149331845
Range	1.42	1.8	1.11	1.32	1.83	1.9	0.28
Minimum	0.17	0.01	0.23	0.14	-1	-0.22	-0.11
Maximum	1.59	1.81	1.34	1.46	0.83	1.68	0.17
Confidence Level(95.0%)	0.41100413	0.296458774	0.574705208	0.329775561	0.465240401	0.287713667	0.370961127

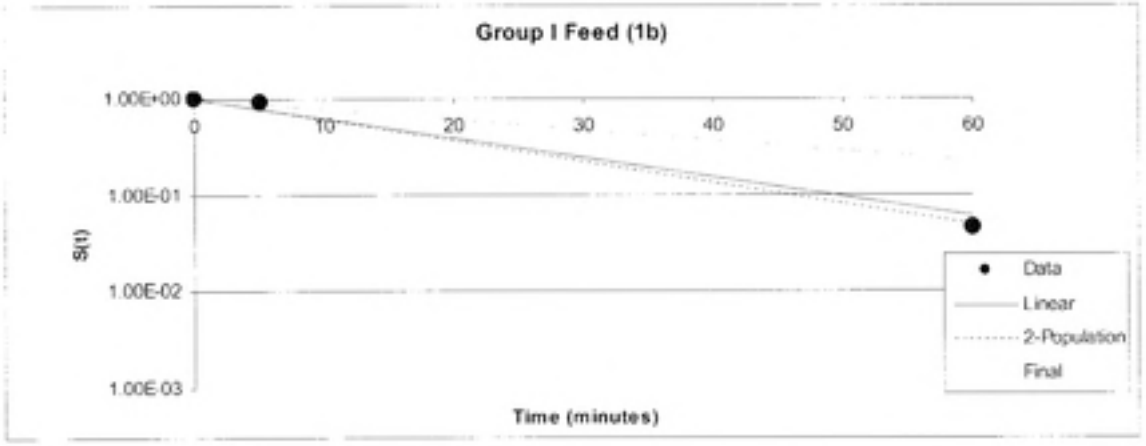
Inactivation Due to Heat ONLY (log_{10} reduction) and (95% CI)							
Isolate #	F+RNA					F+DNA	
	Group I Feed	Group I Batch	MS2 ATCC	Group III Feed	QB ATCC	F+ DNA	F+DNA ATCC
1	0.48 (0.23)	0.31 (0.10)	0.51 (0.23)	2.55 (2.54)	3.28 (3.66)	0.37 (0.68)	-0.05 (-0.04)
2	0.49 (0.75)	0.30 (0.15)	1.79 (1.92)	1,27 (1.13)	4.77 (4.89)	0.26 (0.13)	0.43 (0.72)
3	1.36 (1.68)	0.35 (0.16)	0.01 (0.03)	0.98 (1.01)	4.40 (4.72)	0.03 (0.01)	0.102 (0.05)
4	0.79 (0.91)	0.92 (0.98)	0.26 (0.13)	3.33 (2.64)	3.09 (3.02)	0.36 (0.70)	
5	0.92 (0.96)	0.26 (0.15)	0.15 (0.08)	> 3.86	> 5.81	-0.24 (-0.12)	
6	0.96 (0.46)	0.66 (0.81)		> 4.70	4.21 (4.61)	-0.54 (-0.27)	
7	1.03 (1.01)	0.39 (0.71)		> 4.70	3.39 (3.71)	0.07 (0.03)	
8	0.39 (0.72)	0.92 (0.98)		2.02 (2.01)	> 5.49	1.93 (1.99)	
9	0.45 (0.20)	1.05 (1.03)		> 3.78		0.46 (0.25)	
10		0.96 (0.96)		> 2.95		-0.27 (-0.13)	
11		1.08 (1.02)				-0.41 (-0.21)	
12		0.78 (0.91)				0.27 (0.13)	
13		0.46 (0.21)				-0.04 (-0.02)	
14						-0.07 (-0.02)	
Mean	0.763333333	0.649230769	0.544	3.014	4.305	0.173076923	0.160666667
Standard Deviation	0.332340187	0.314601906	0.720194418	1.311218263	1.018935018	0.615878568	0.24531884
Range	0.97	0.82	1.78	>3.72	>2.72	2.47	0.48
Minimum	0.39	0.26	0.01	0.98	3.09	-0.54	-0.05
Maximum	1.36	1.08	1.79	>4.7	>5.81	1.93	0.43
Confidence Level(95.0%)	0.255459447	0.190112026	0.89424133	0.937989755	0.851850384	0.372171687	0.609406206

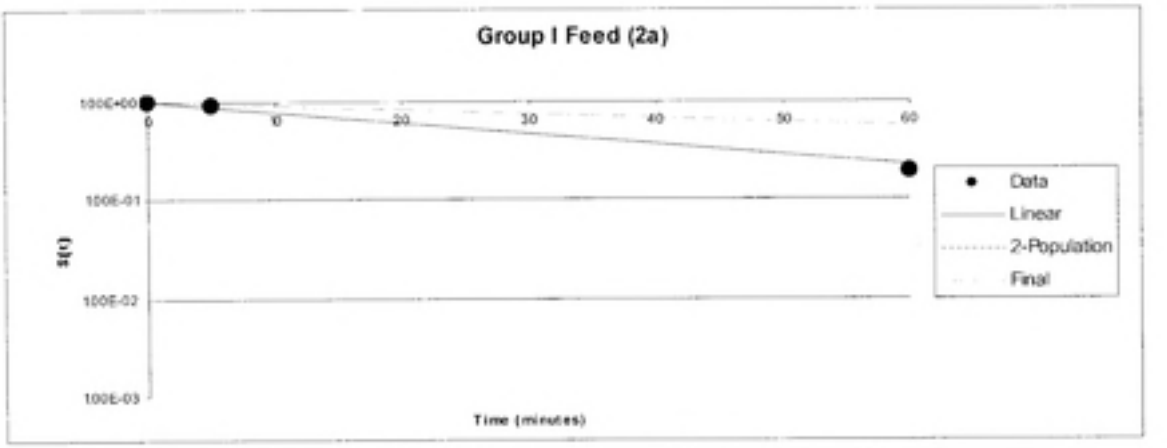
* The ">" sign indicates there was no detectable coliphage after 60 minutes. For calculation purposes only, it was assumed that the concentration was 50 pfu/ml. However, because fewer that this were detectable, the coliphage inactivated to a greater extent than indicated by the calculation.

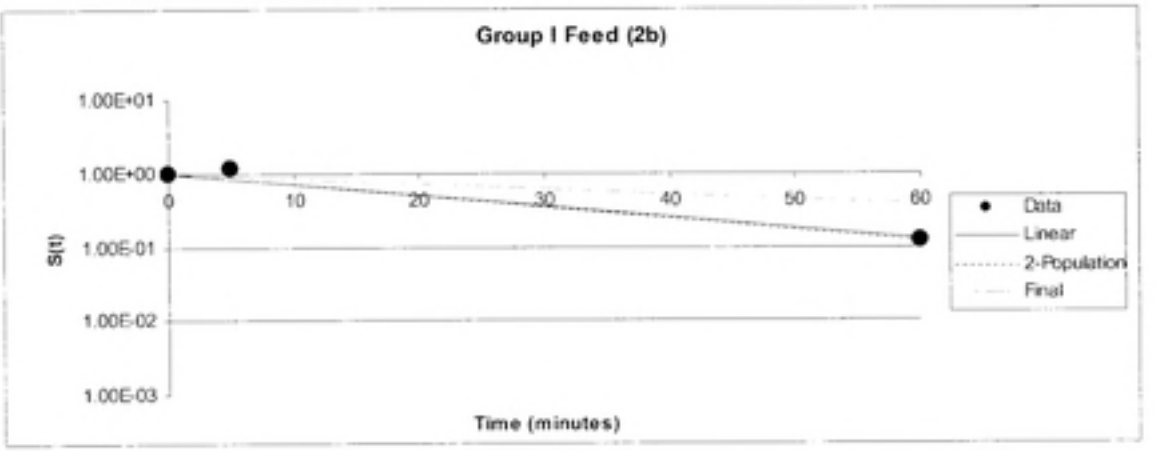
APPENDIX 6. Inactivation Charts for All Isolates

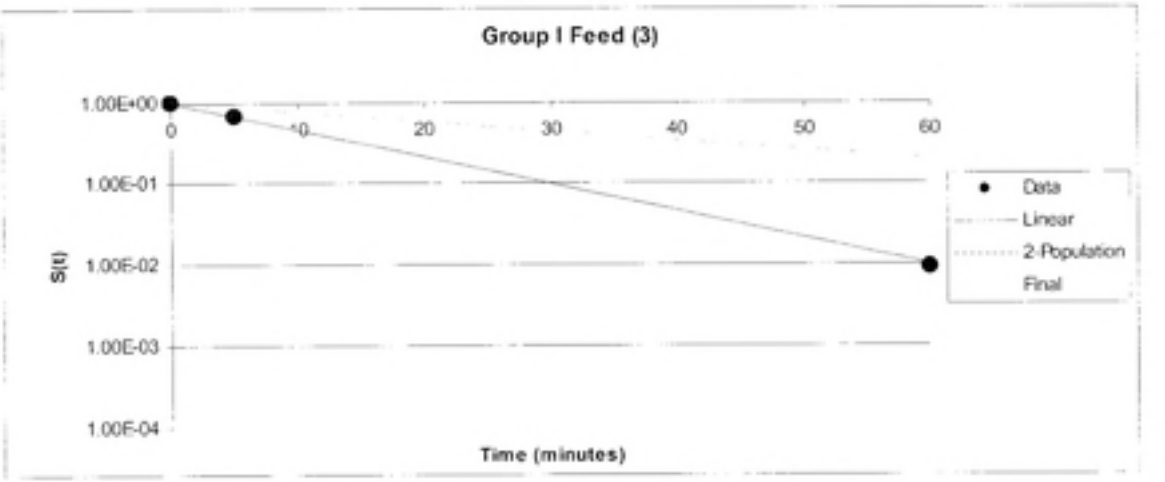
Data = Log_{10} inactivation of the isolate at 5 or 60 minutes
Linear = The line illustrating 1st Order Kinetics
2-Population = The best fit curve of the data points in a 2-population model
Final = The line illustrating the inactivation of the isolates at room temperature (~25 °C) after 60-75 minutes

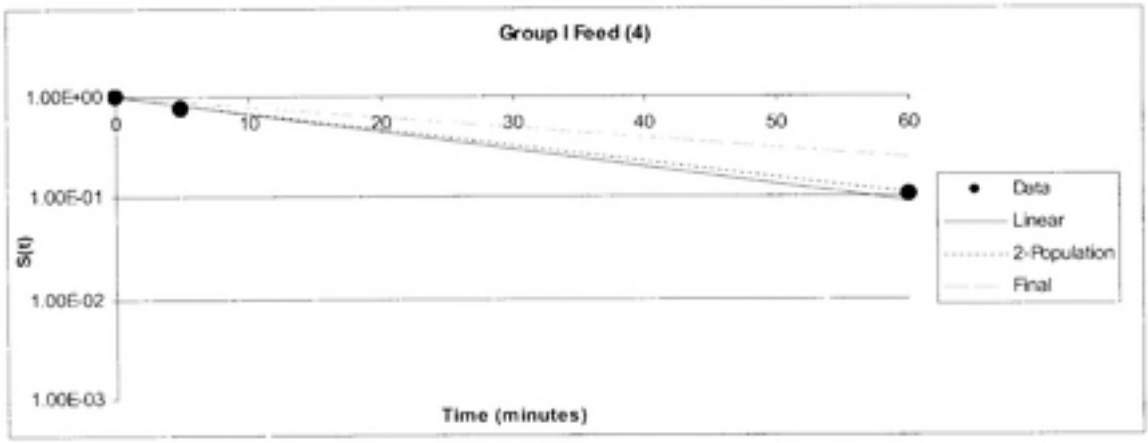


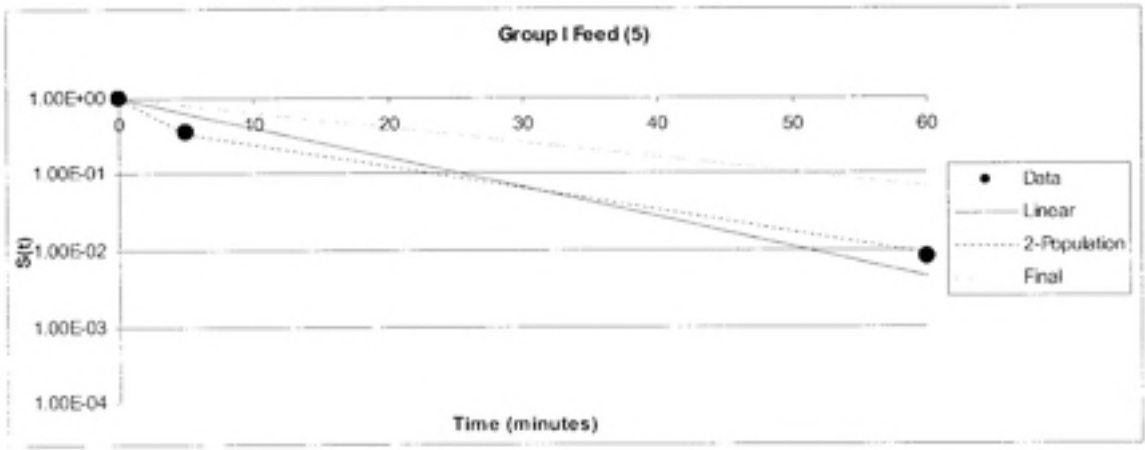


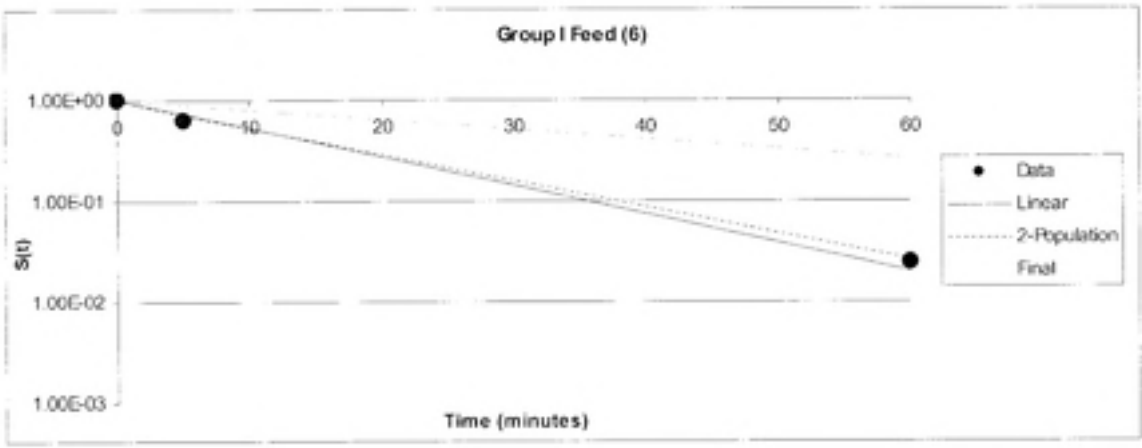

Group I Feed (2a)
S(t)
Time (minutes)
Data
Linear
2-Population
Final

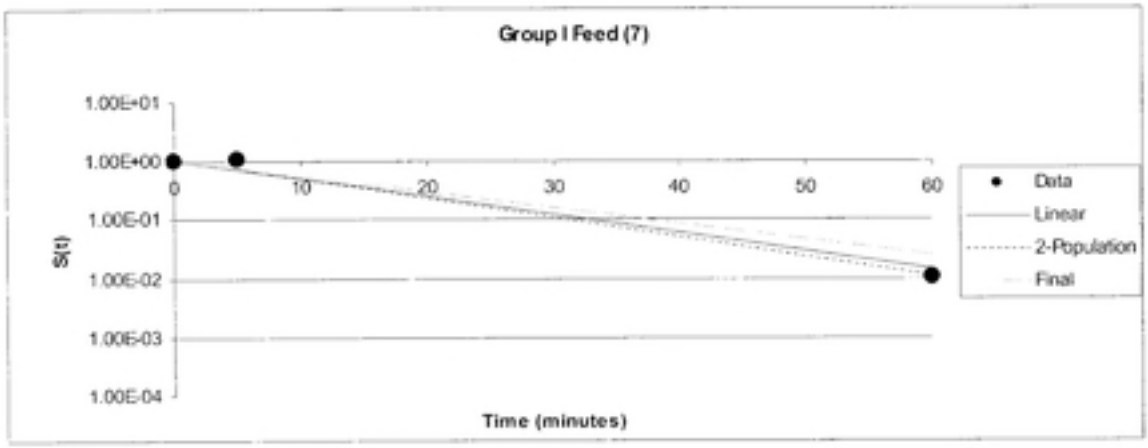

Group I Feed (2b)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

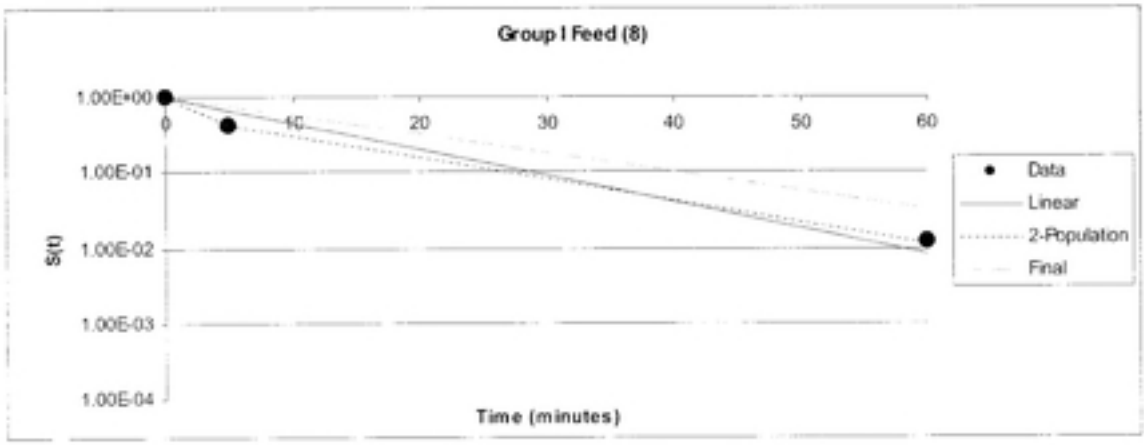

Group I Feed (3)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

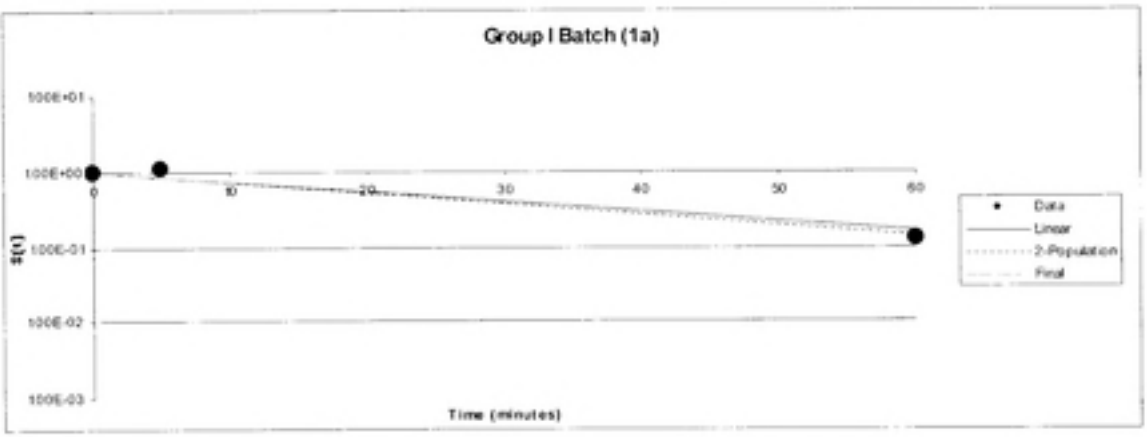

Group I Feed (4)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

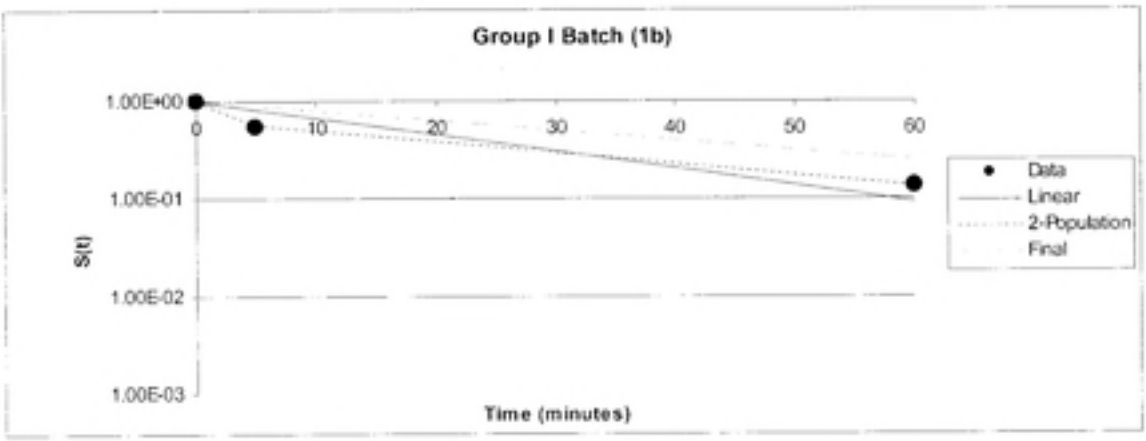

Group I Feed (5)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

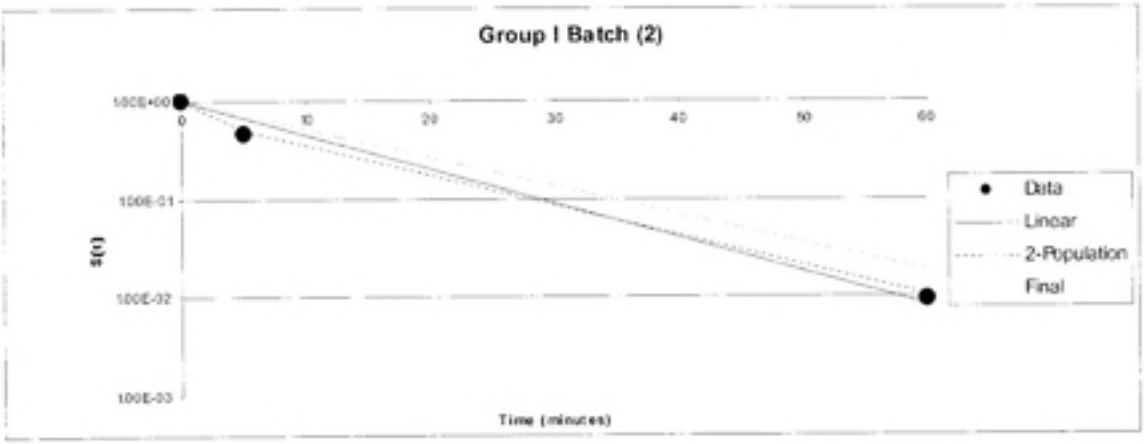

Group I Feed (6)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

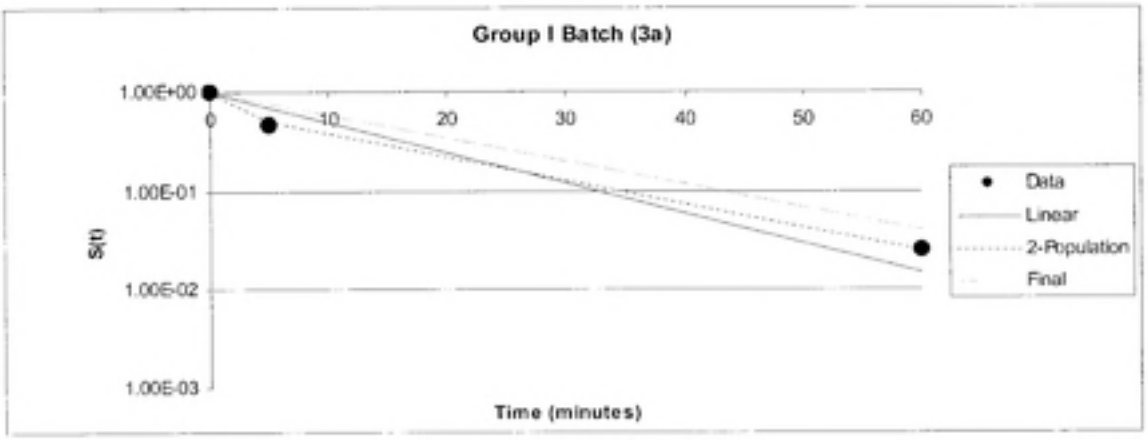

Group I Feed (7)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

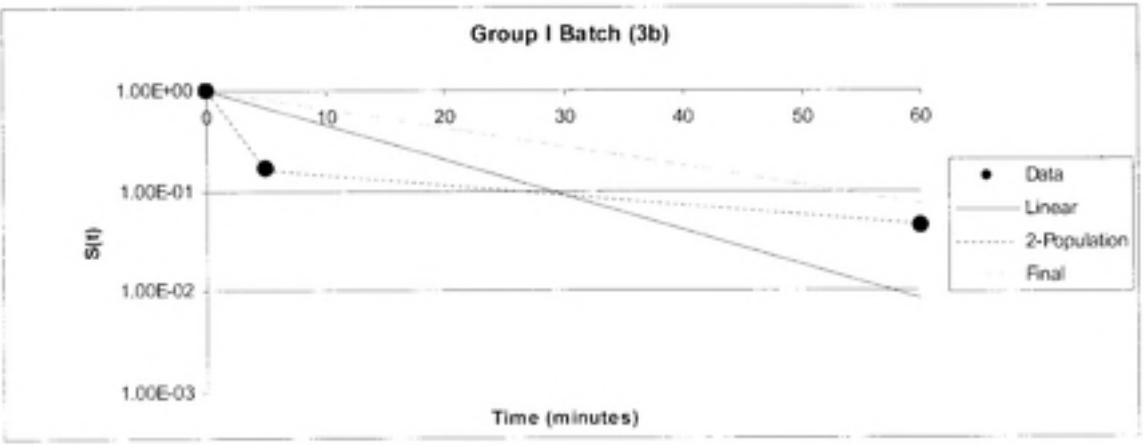

Group I Feed (8)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

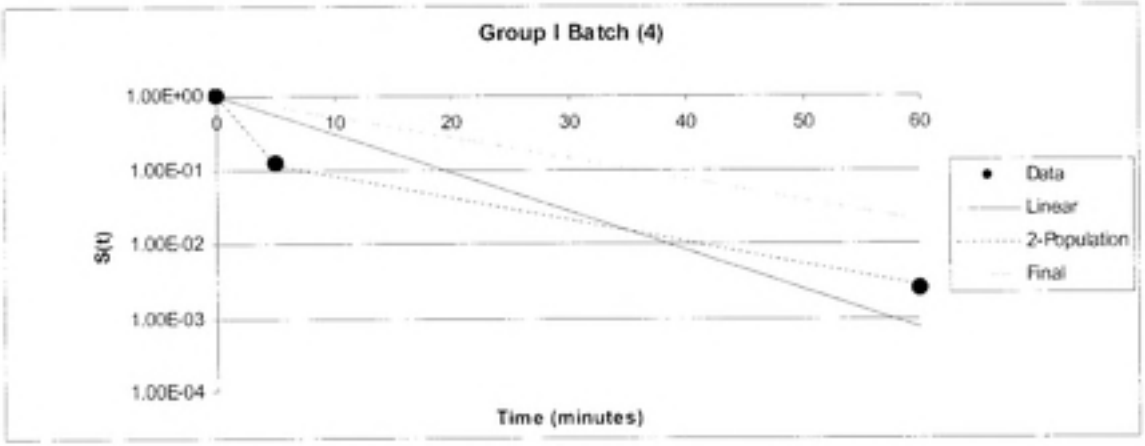

Group I Batch (1a)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

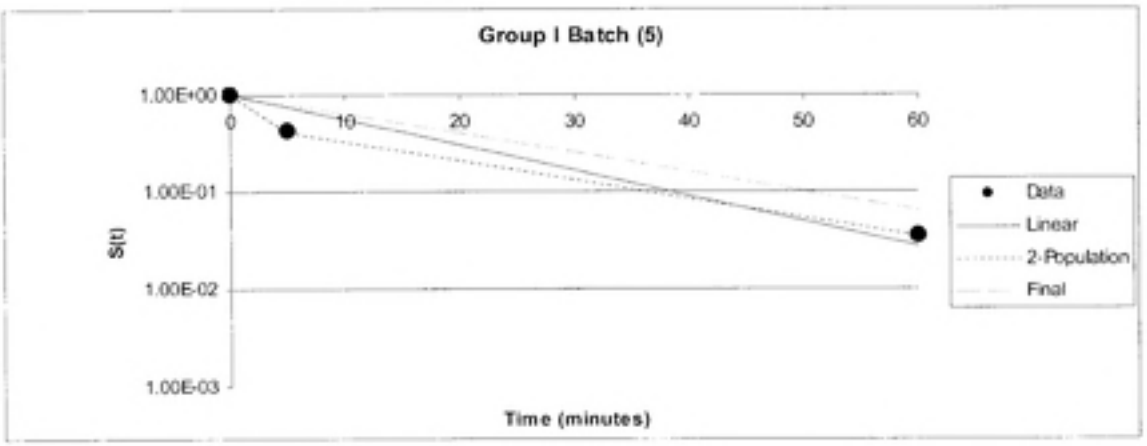

Group I Batch (1b)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

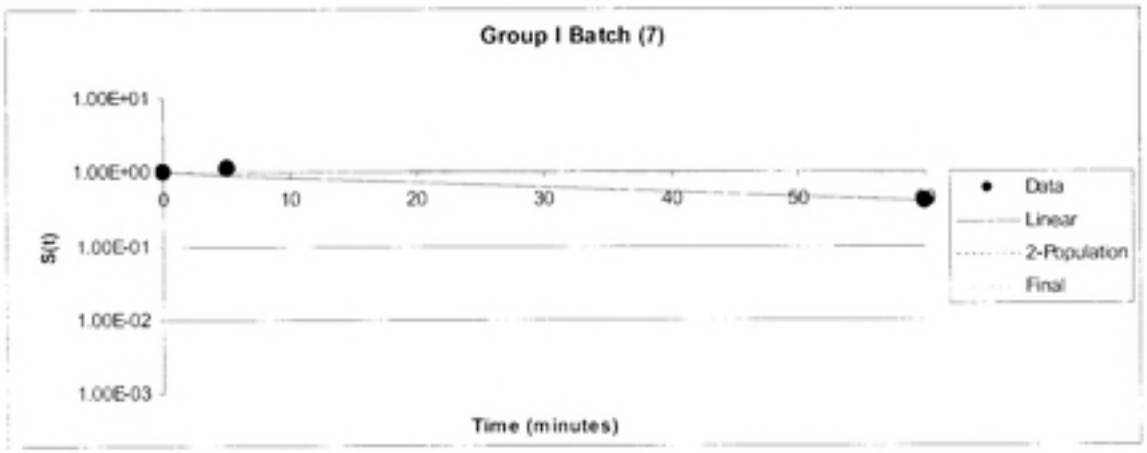

Group I Batch (2)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

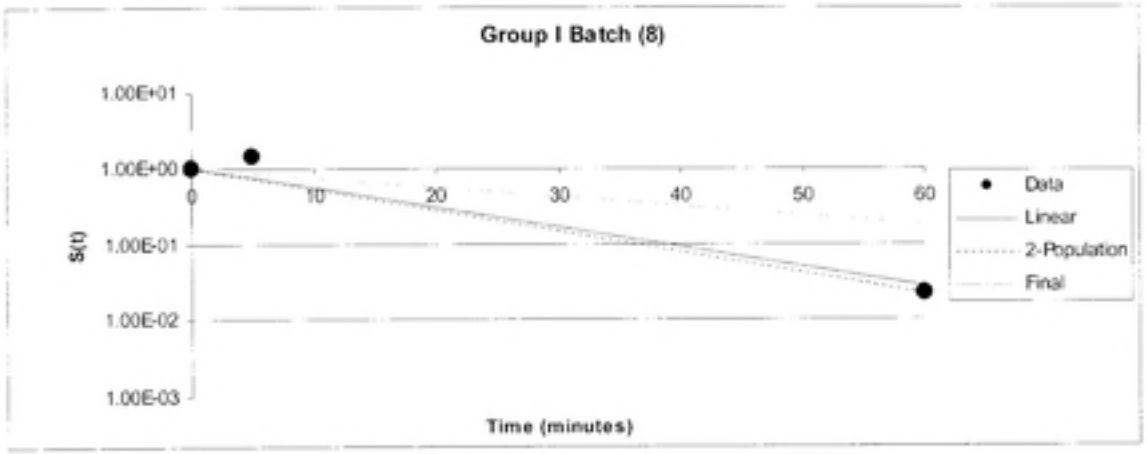

Group I Batch (3a)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

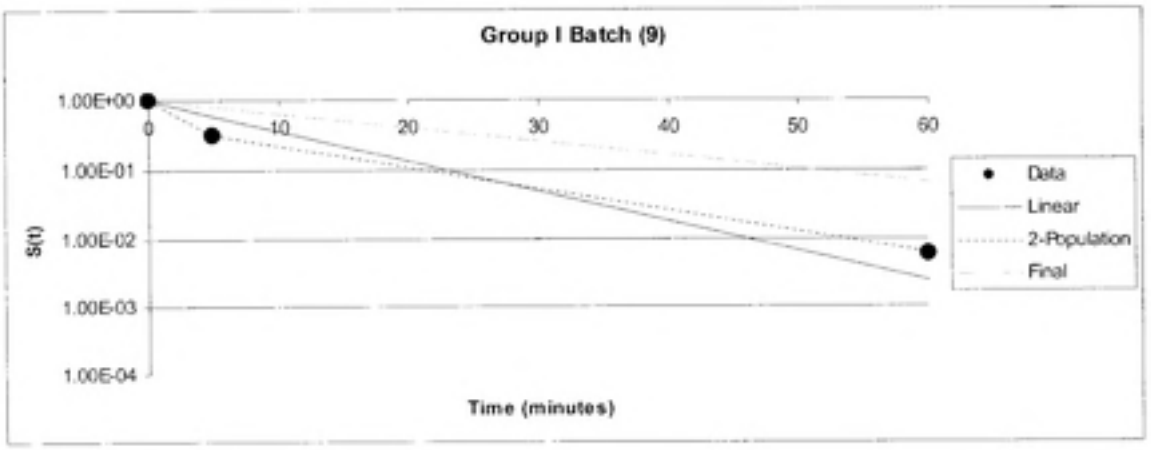

Group I Batch (3b)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

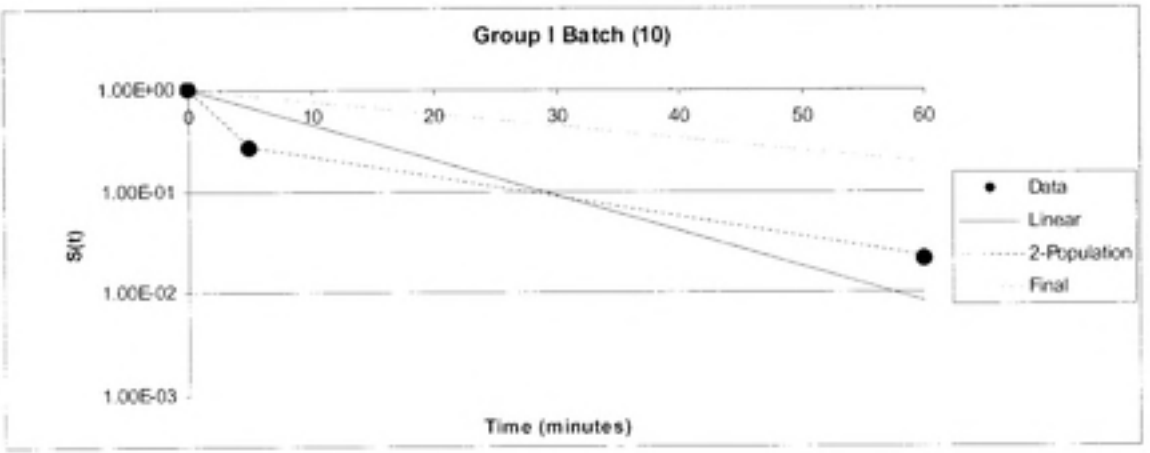

Group I Batch (4)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

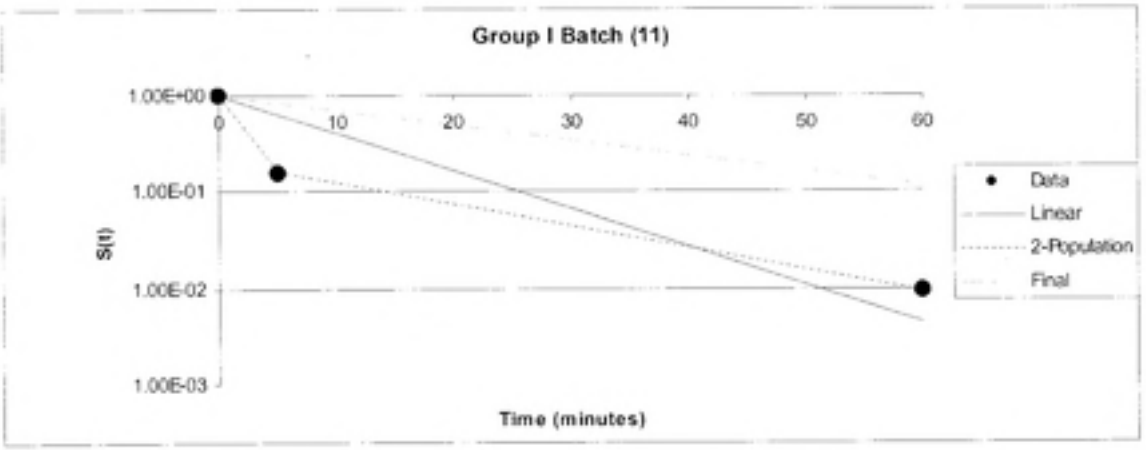

Group I Batch (5)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

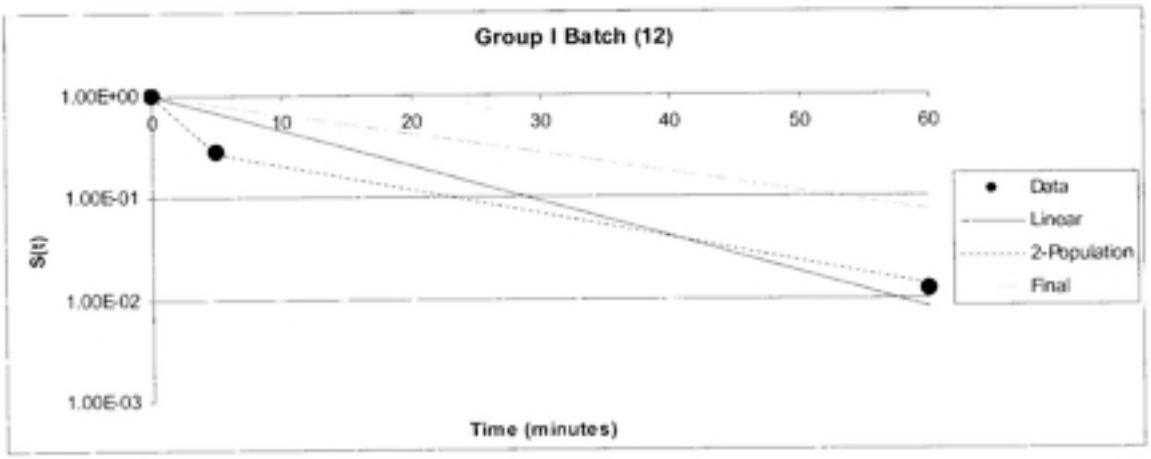

Group I Batch (7)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
Time (minutes)
Data
Linear
2-Population
Final

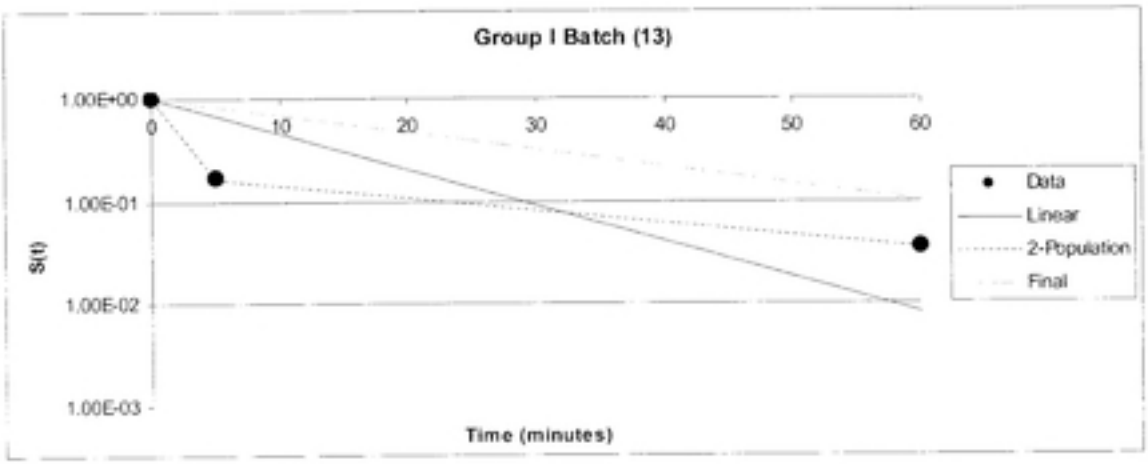

Group I Batch (8)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

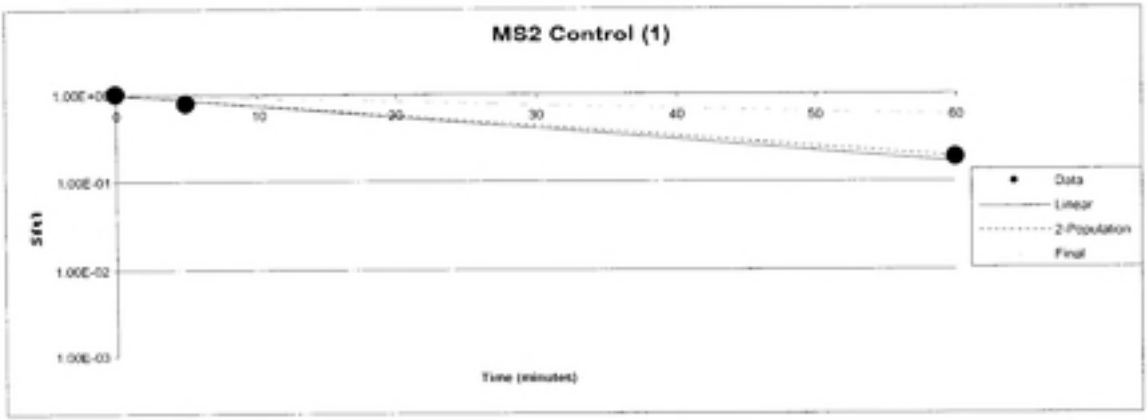

Group I Batch (9)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

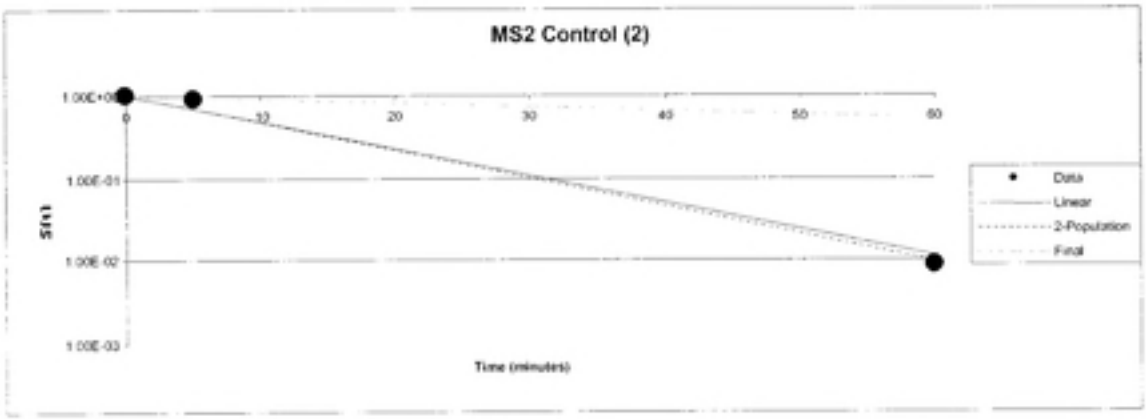

Group I Batch (10)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

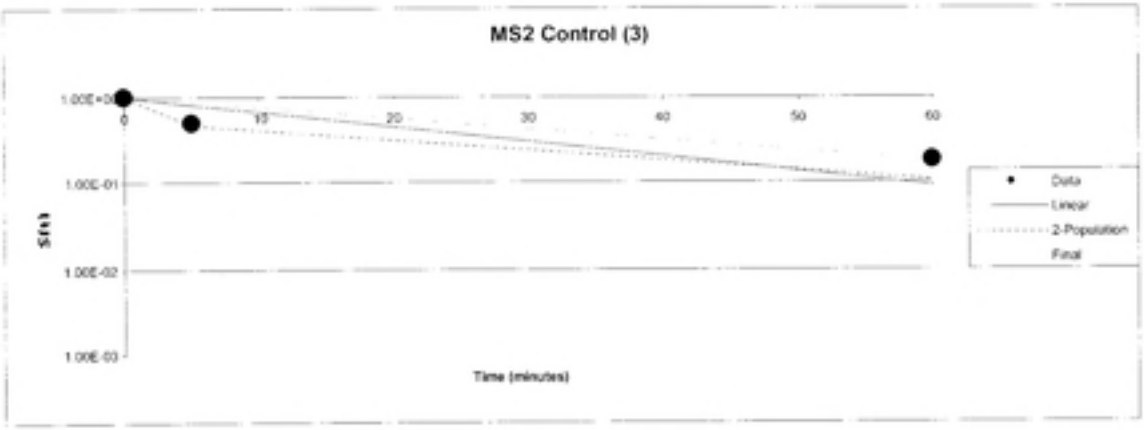

Group I Batch (11)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

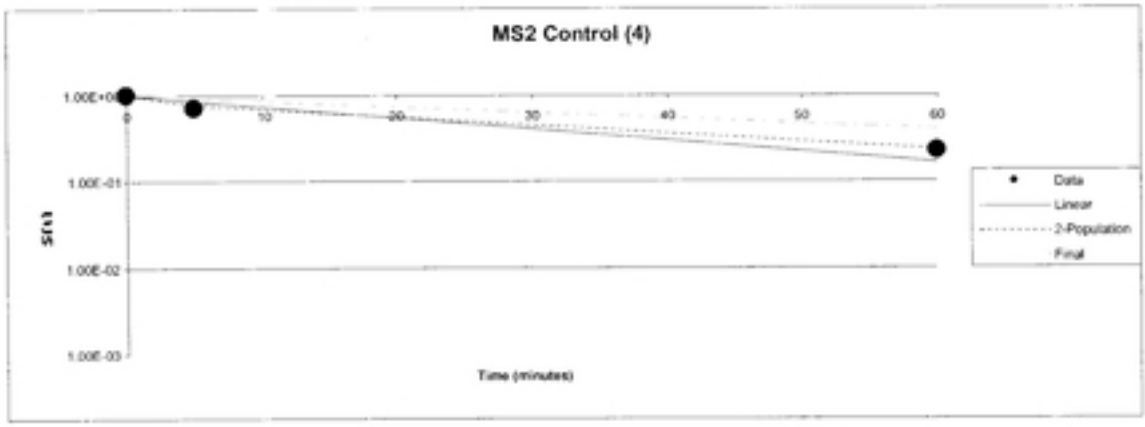

Group I Batch (12)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

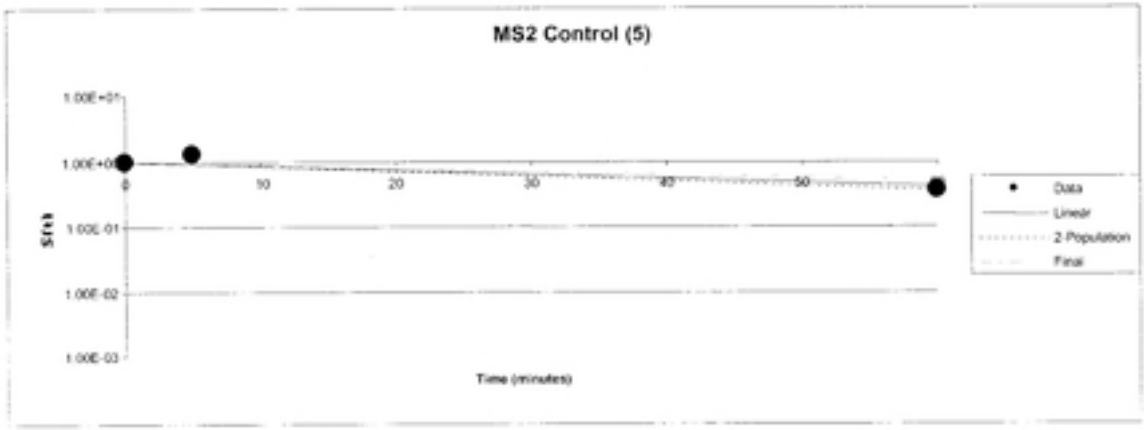

Group I Batch (13)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

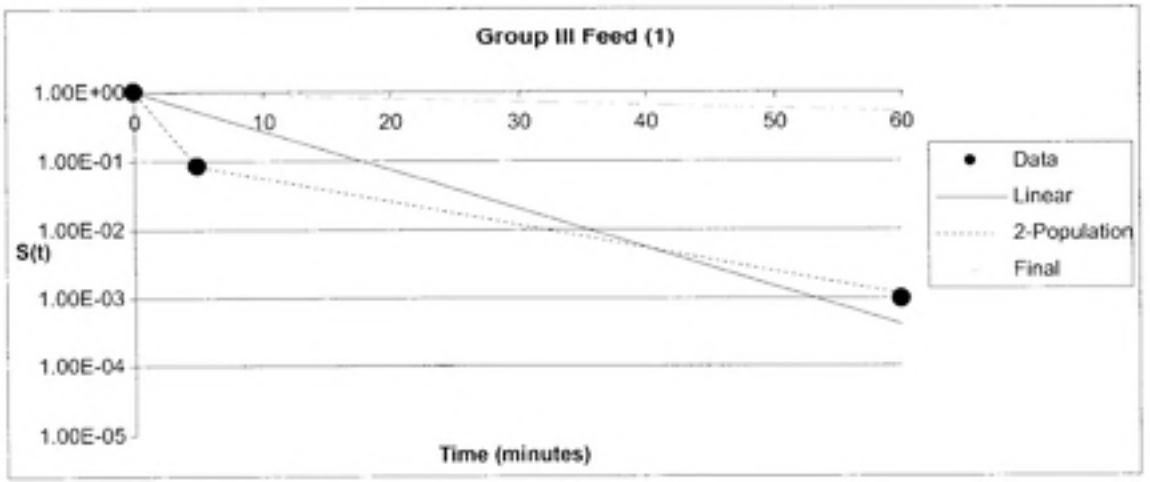

MS2 Control (1)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(n)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

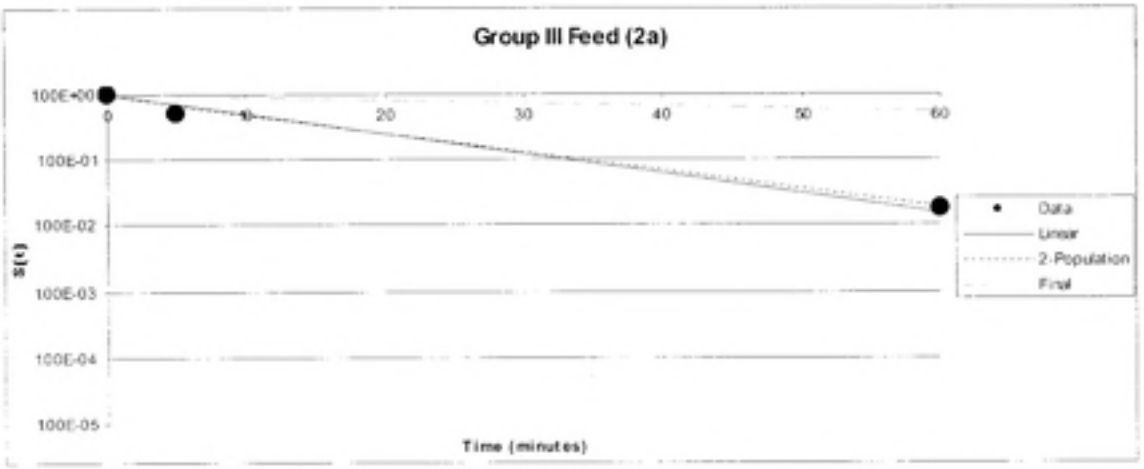

MS2 Control (2)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(n)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

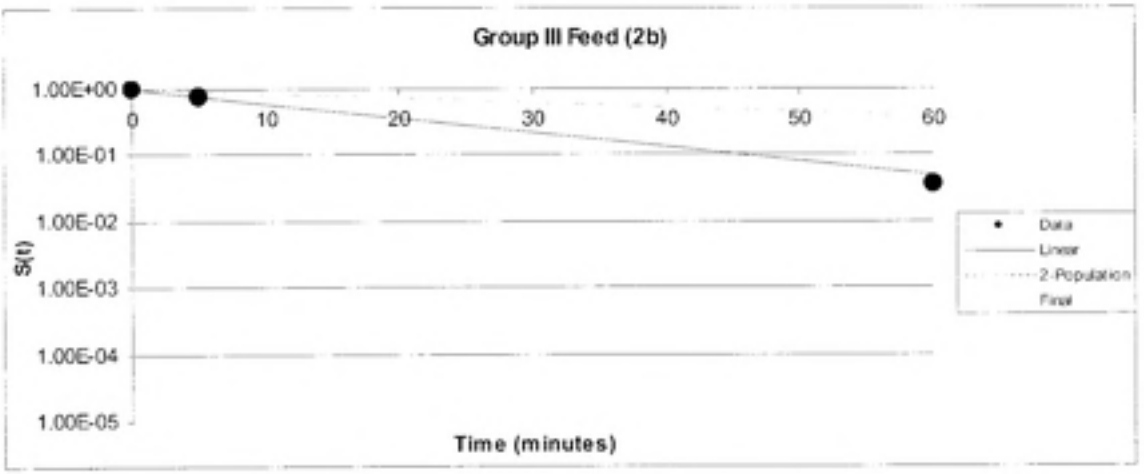

MS2 Control (3)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(n)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

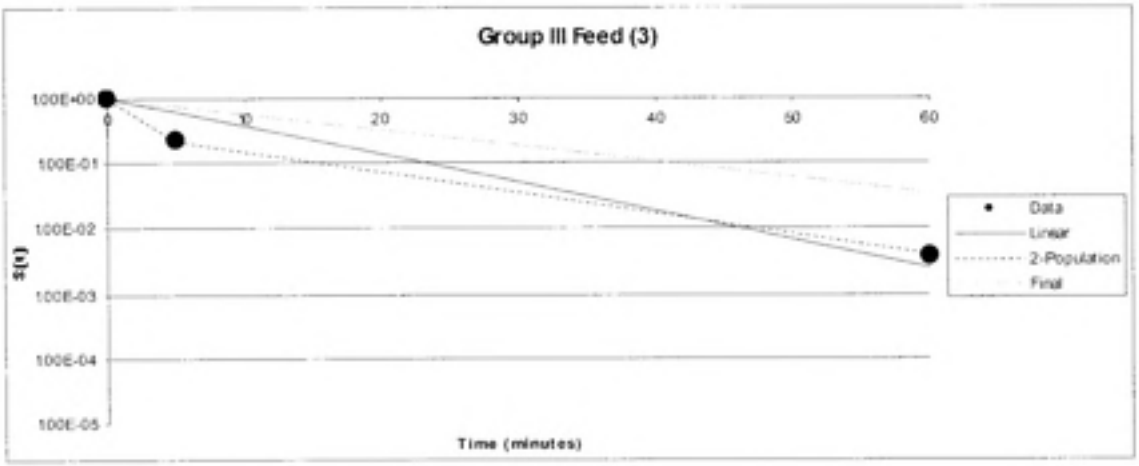

MS2 Control (4)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

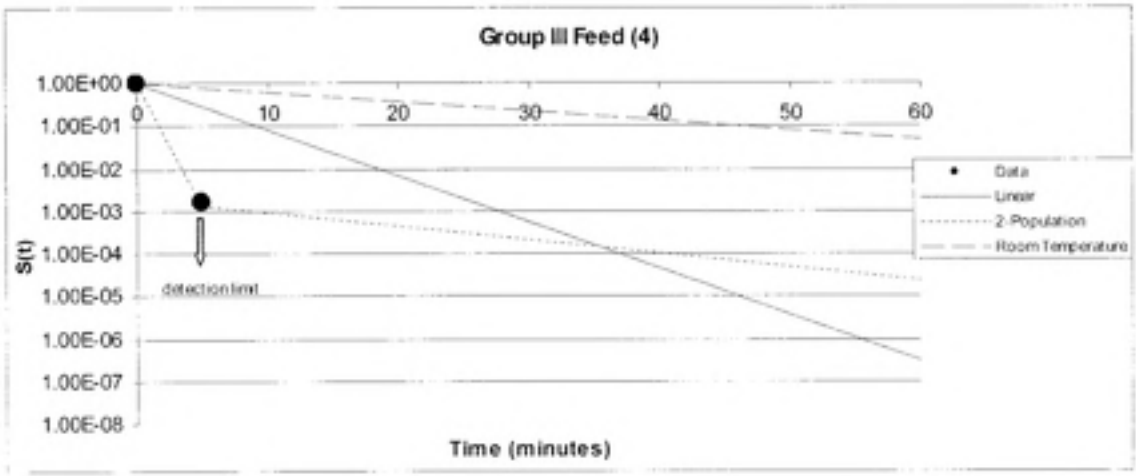

MS2 Control (5)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
Time (minutes)
Data
Linear
2 Population
Final

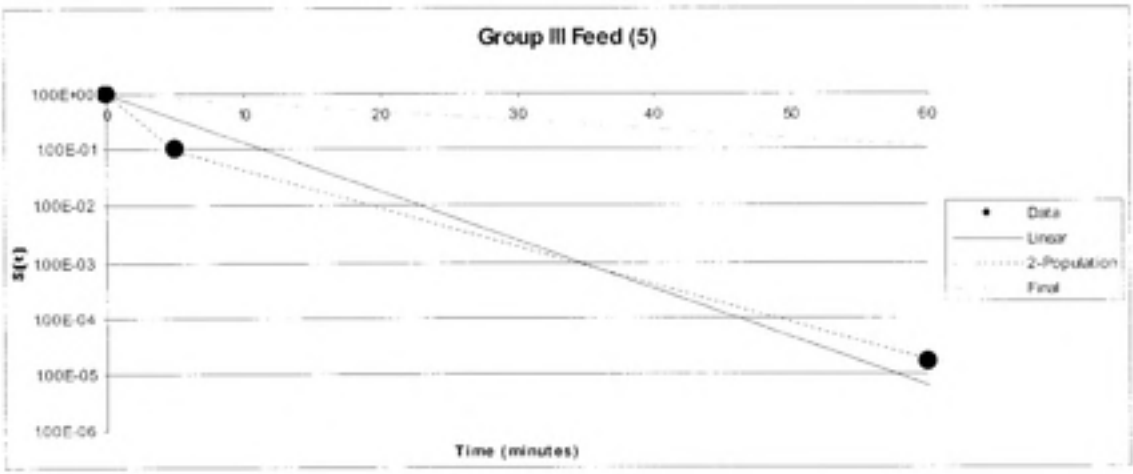

Group III Feed (1)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

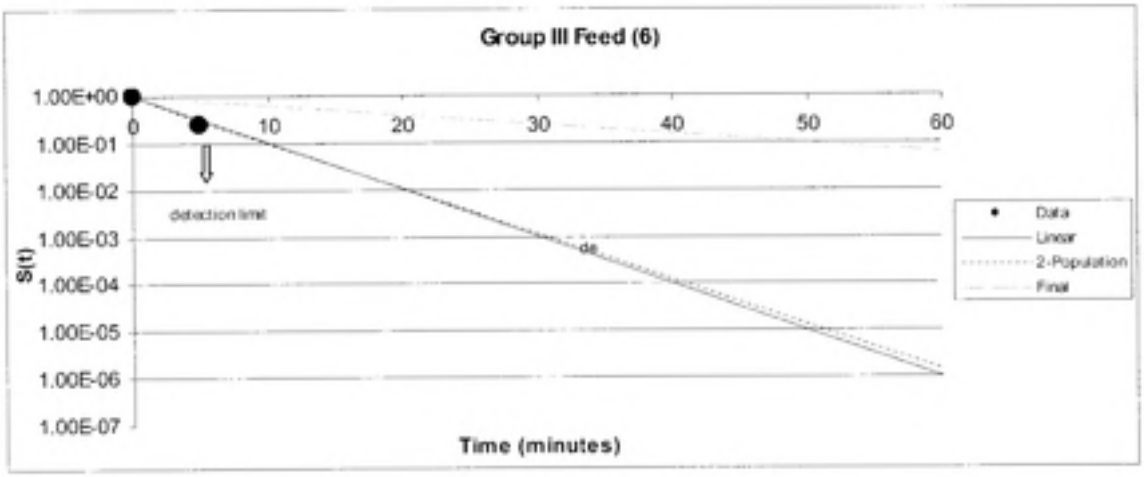

Group III Feed (2a)
S(t)
Time (minutes)
Data
Linear
2-Population
Final

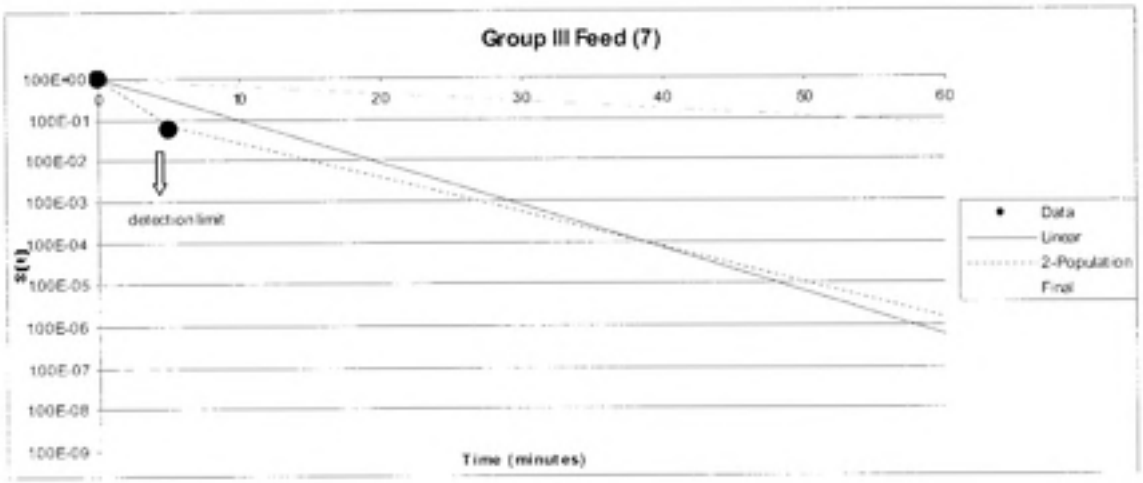

Group III Feed (2b)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

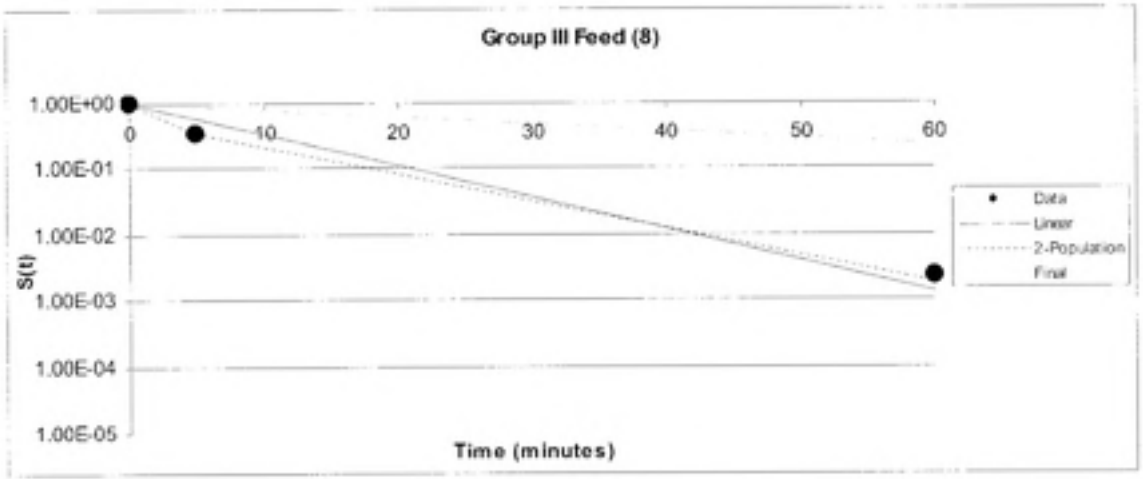

Group III Feed (3)
100E+00
100E-01
100E-02
100E-03
100E-04
100E-05
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

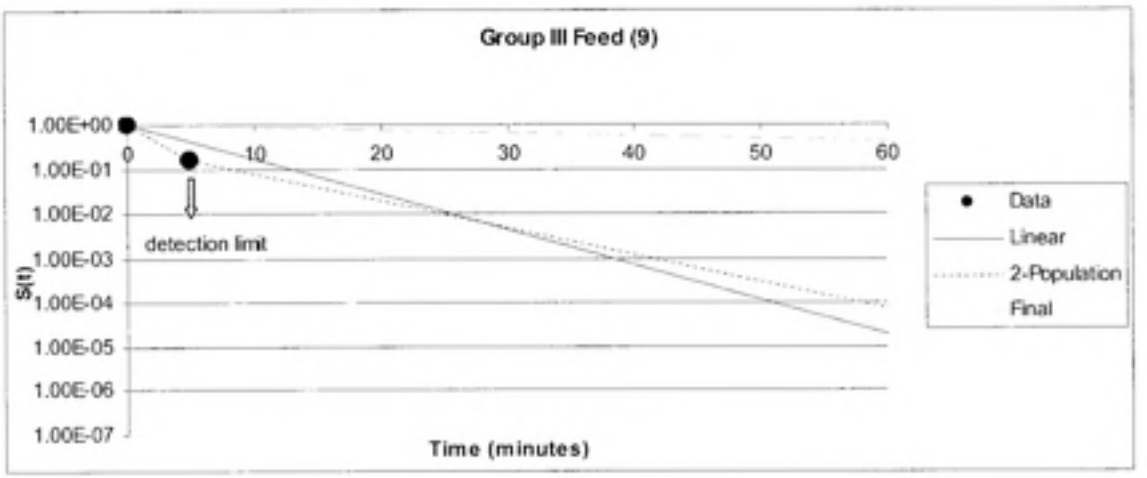

Group III Feed (4)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
S(t)
0
10
20
30
40
50
60
detection limit
Time (minutes)
Data
Linear
2-Population
Room Temperature

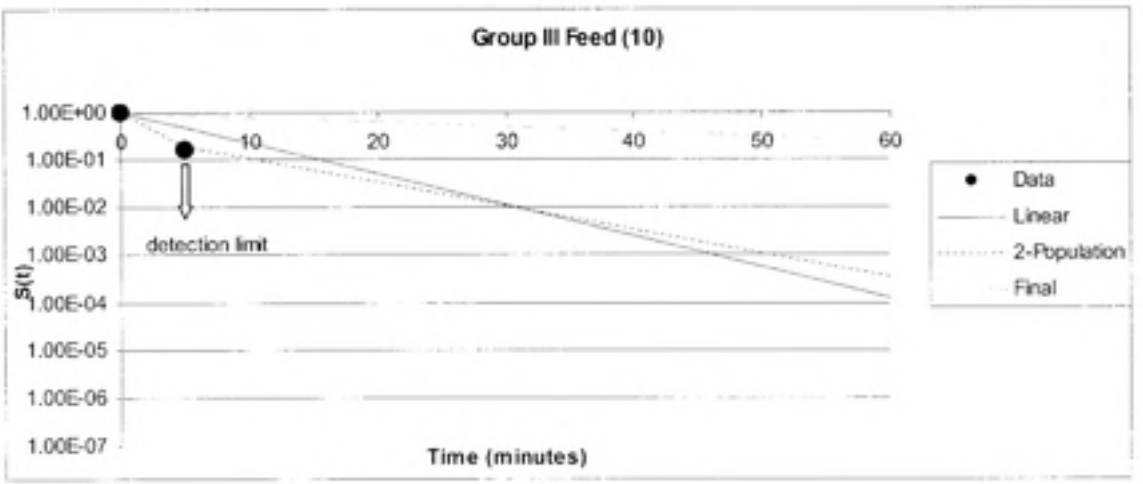

Group III Feed (5)
100E+00
100E-01
100E-02
100E-03
100E-04
100E-05
100E-06
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

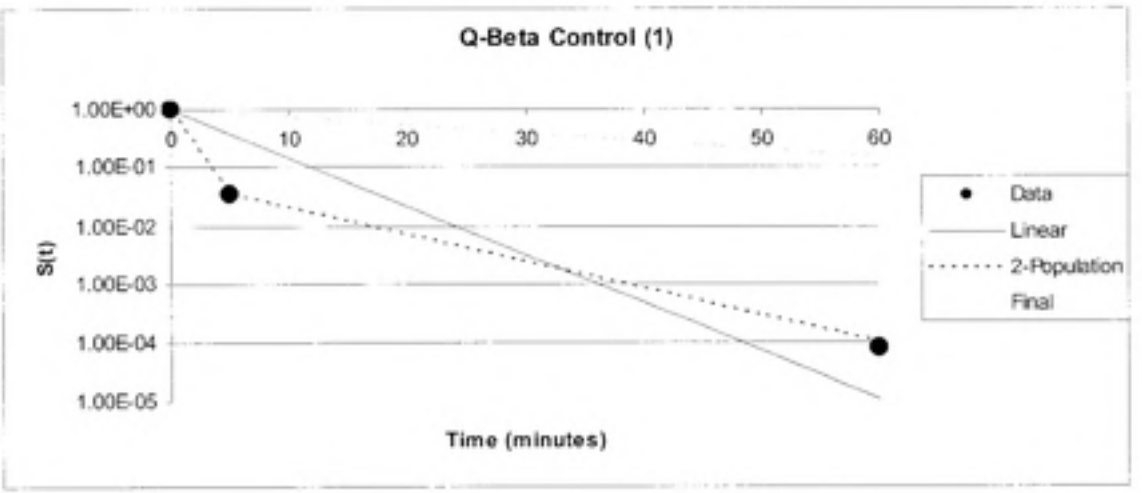

Group III Feed (6)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
S(t)
0
10
20
30
40
50
60
detection limit
Data
Linear
2-Population
Final
Time (minutes)

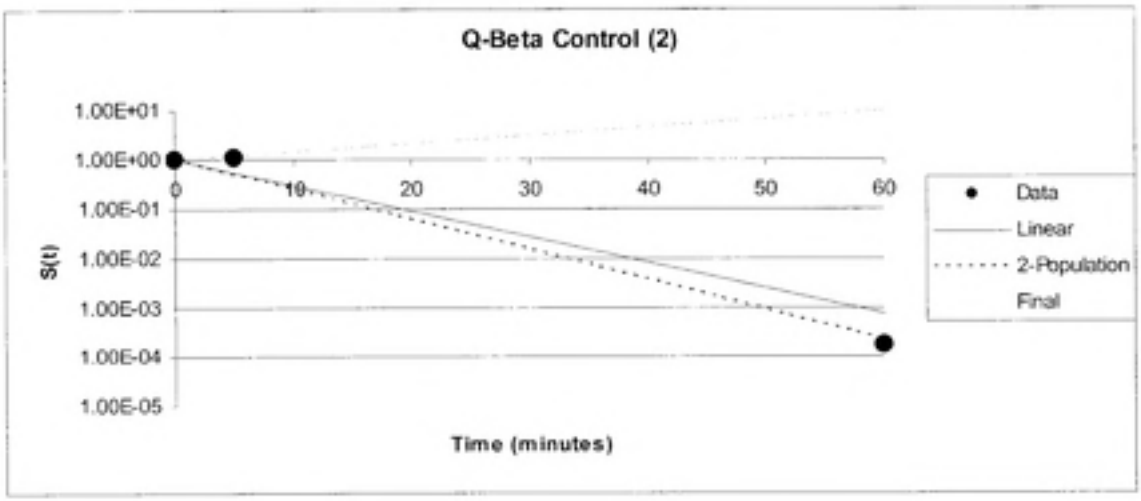

Group III Feed (7)
S(t)
detection limit
Data
Linear
2-Population
Final
Time (minutes)

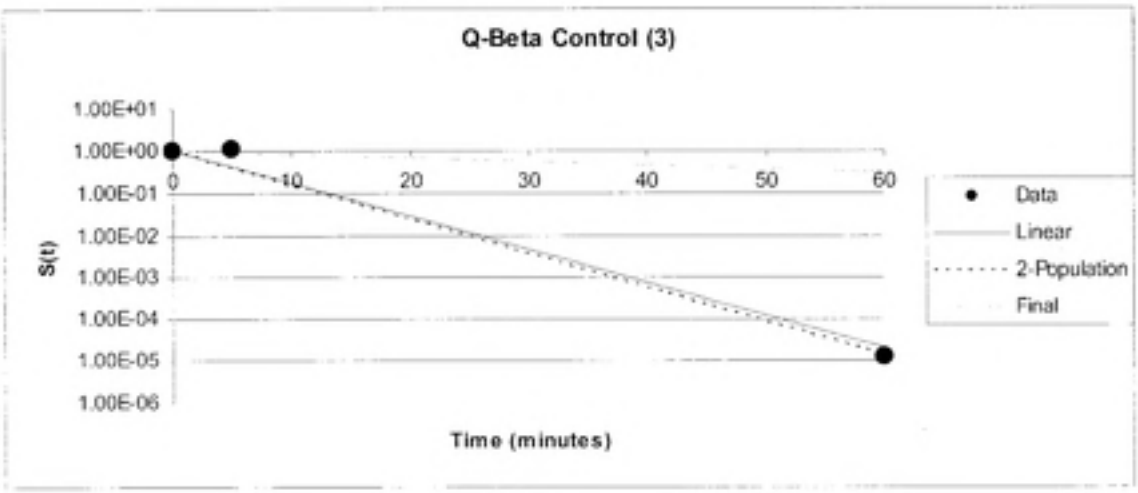

Group III Feed (8)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
S(t)
0
10
20
30
40
50
60
Data
Linear
2-Population
Final
Time (minutes)

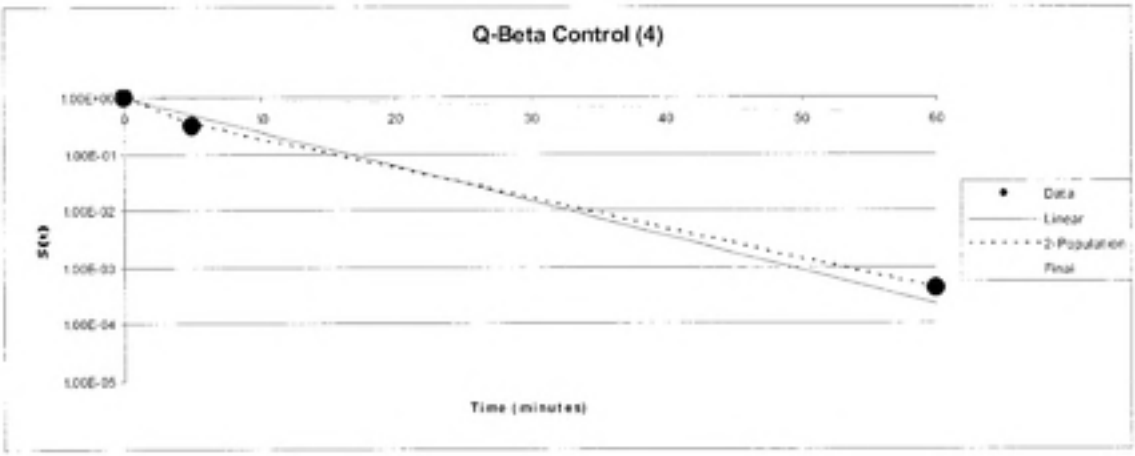

Group III Feed (9)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
S(t)
0
10
20
30
40
50
60
detection limit
Time (minutes)
Data
Linear
2-Population
Final

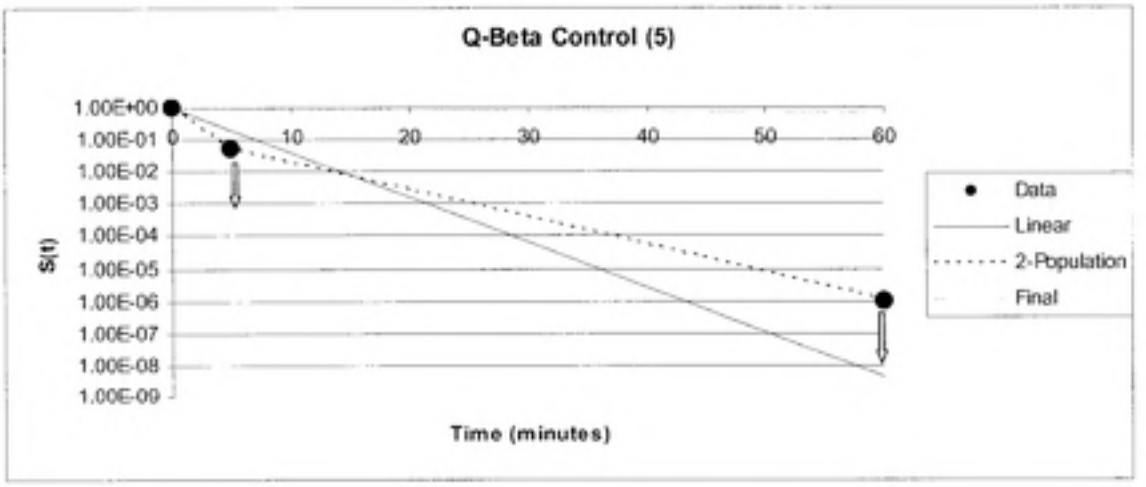

Group III Feed (10)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
S(t)
0
10
20
30
40
50
60
detection limit
Time (minutes)
Data
Linear
2-Population
Final

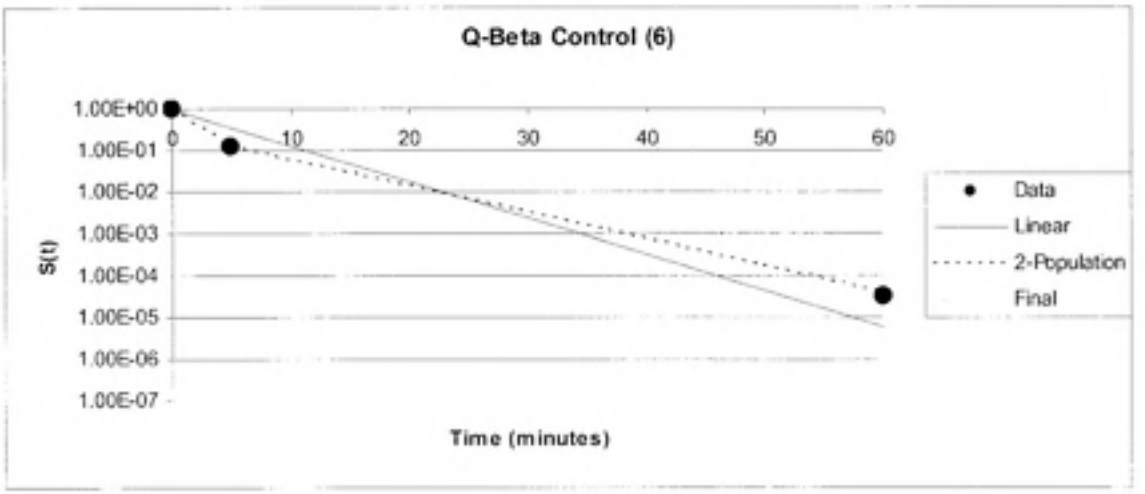

Q-Beta Control (1)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

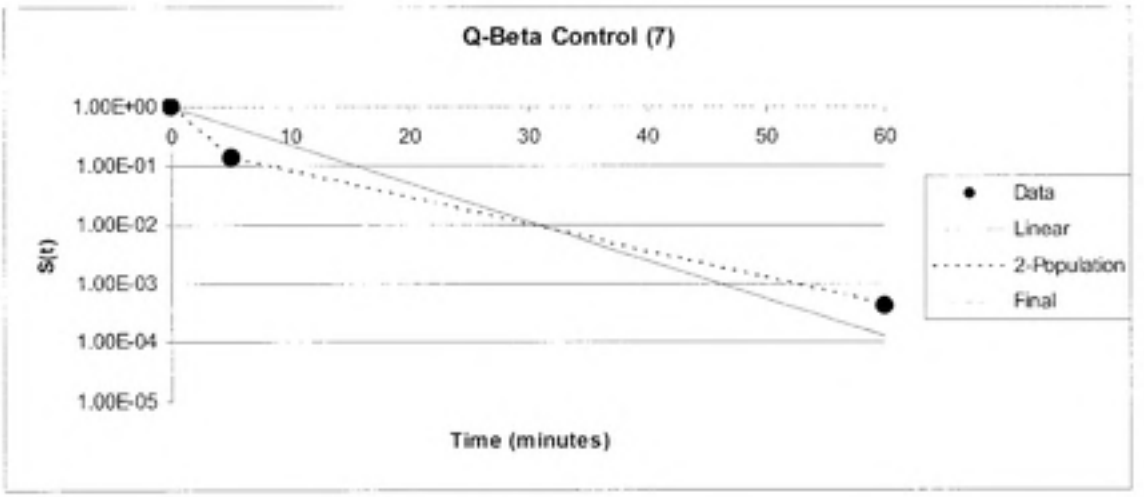

Q-Beta Control (2)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

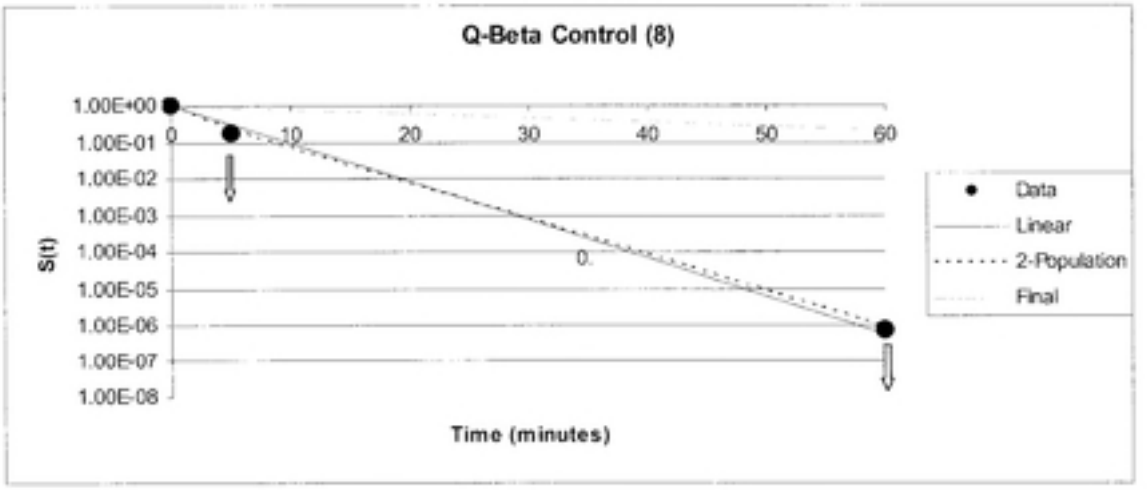

Q-Beta Control (3)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

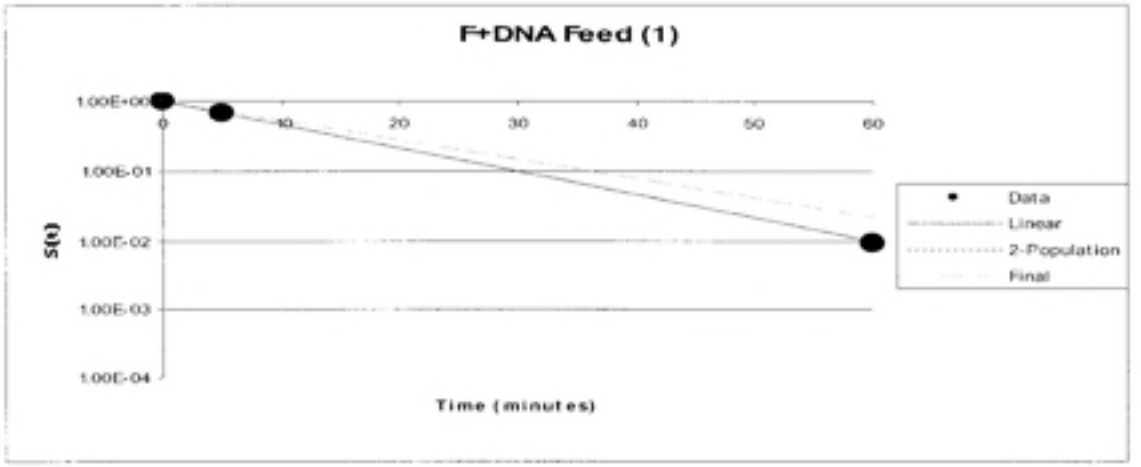

Q-Beta Control (4)
S(t)
Time (minutes)
Data
Linear
2-Population
Final

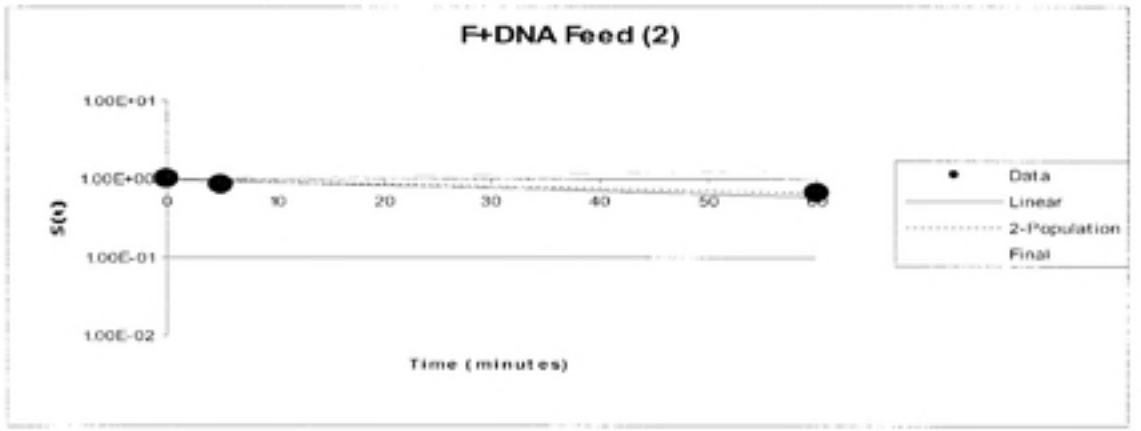

Q-Beta Control (5)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
1.00E-09
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

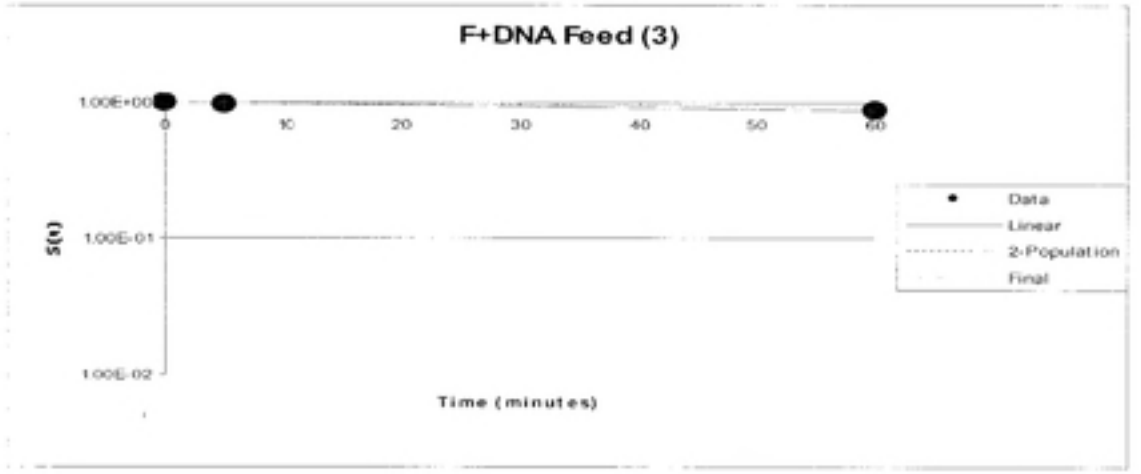

Q-Beta Control (6)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

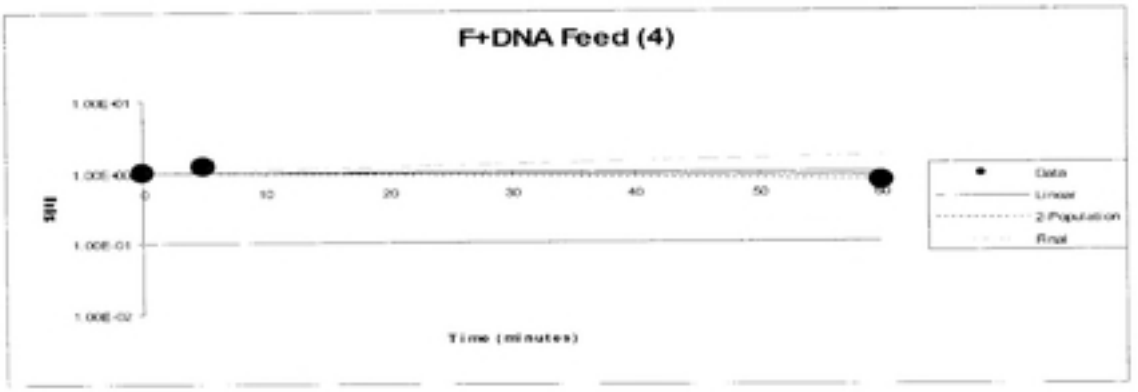

Q-Beta Control (7)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

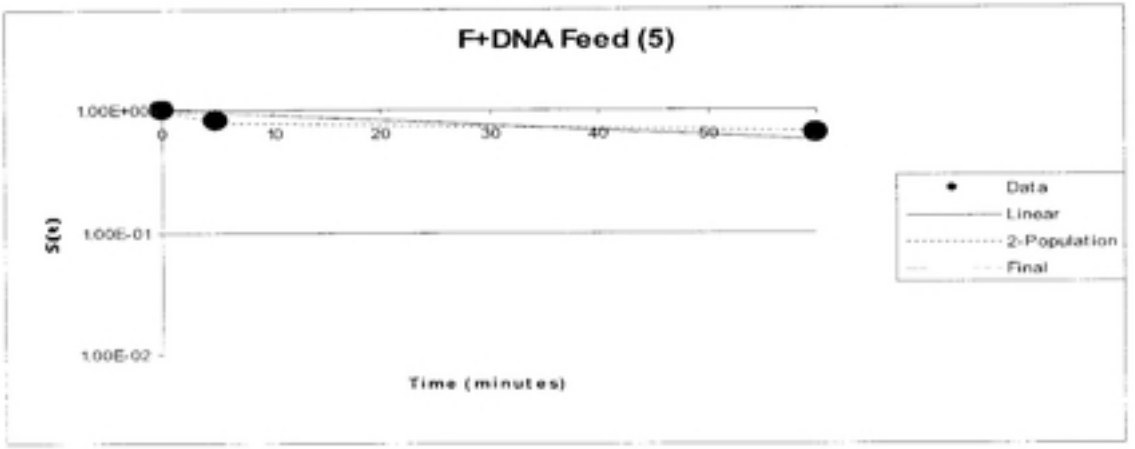

Q-Beta Control (8)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

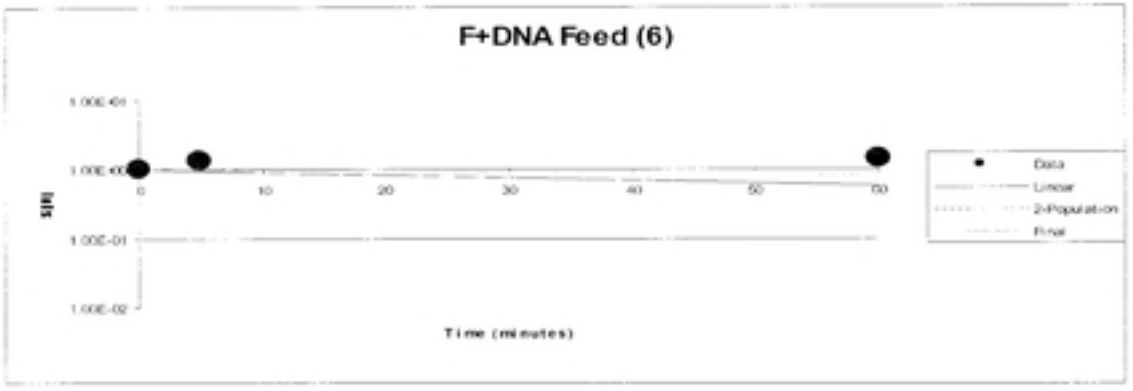

F+DNA Feed (1)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

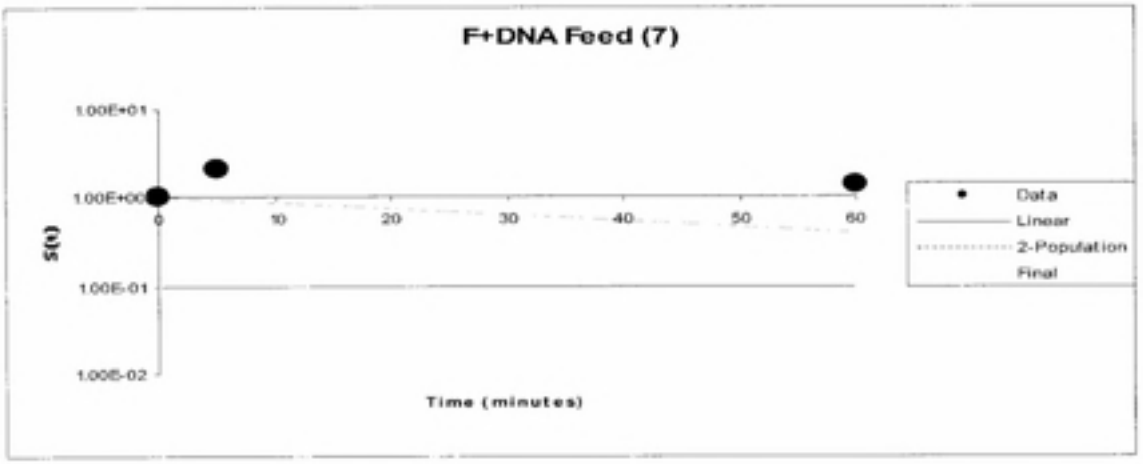

F+DNA Feed (2)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
S(t)
0
10
20
30
40
50
Time (minutes)
Data
Linear
2-Population
Final

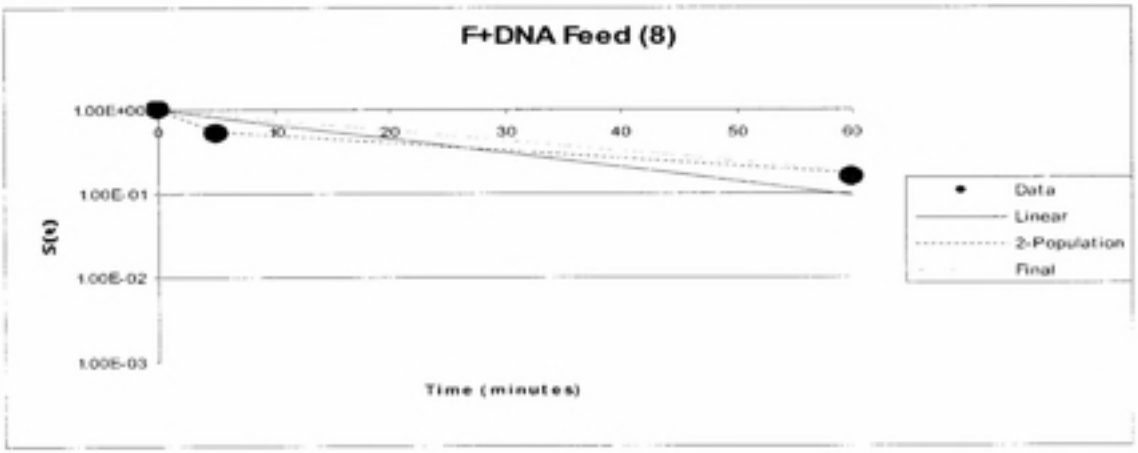

F+DNA Feed (3)
1.00E+00
1.00E-01
1.00E-02
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

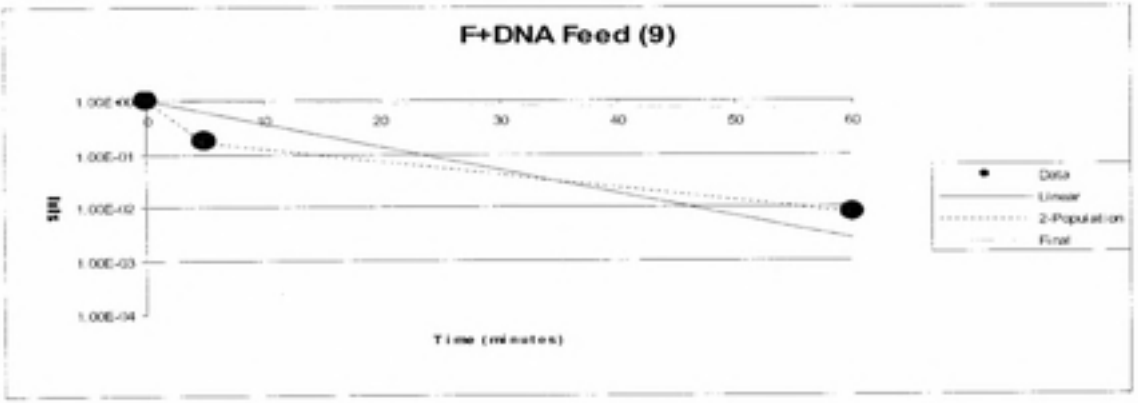

F+DNA Feed (4)
S(t)
1.00E-01
1.00E-02
0
10
20
30
40
50
Time (minutes)
Data
Linear
2-Population
Final

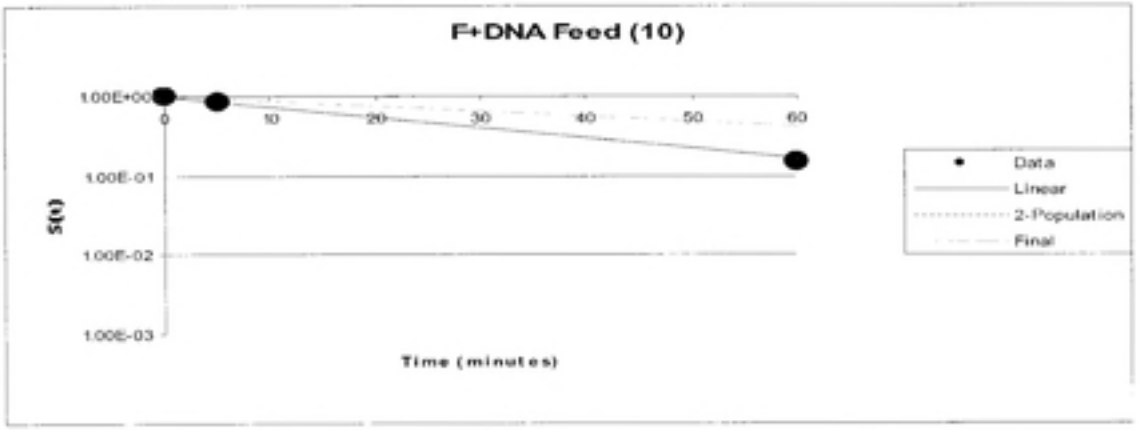

F+DNA Feed (5)
S(t)
1.00E+00
1.00E-01
1.00E-02
0
10
20
30
40
50
Time (minutes)
Data
Linear
2-Population
Final

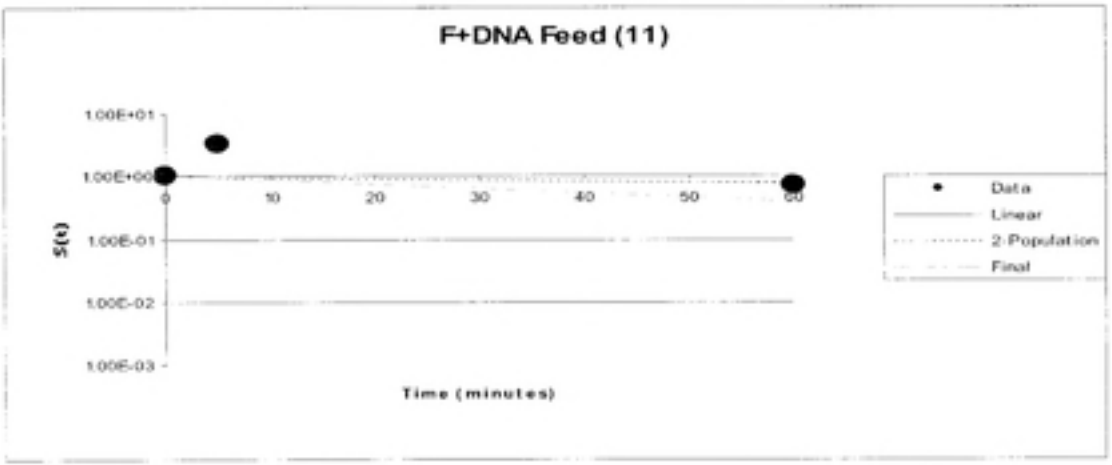

F+DNA Feed (6)
S(t)
1.00E-01
1.00E-02
0
10
20
30
40
50
Time (minutes)
Data
Linear
2-Population
Final


F+DNA Feed (7)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final


F+DNA Feed (8)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

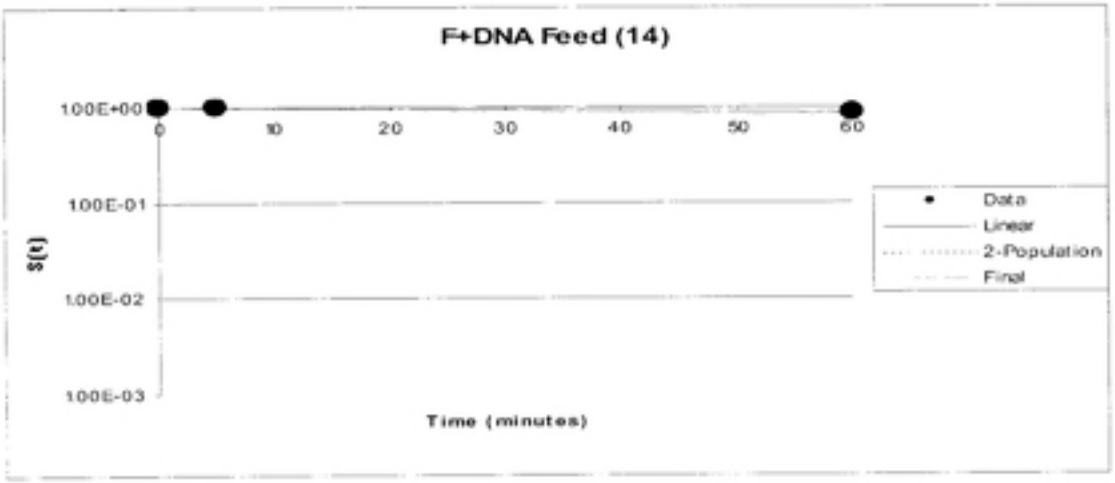

F+DNA Feed (9)
1.00E-01
1.00E-02
1.00E-03
1.00E-04
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

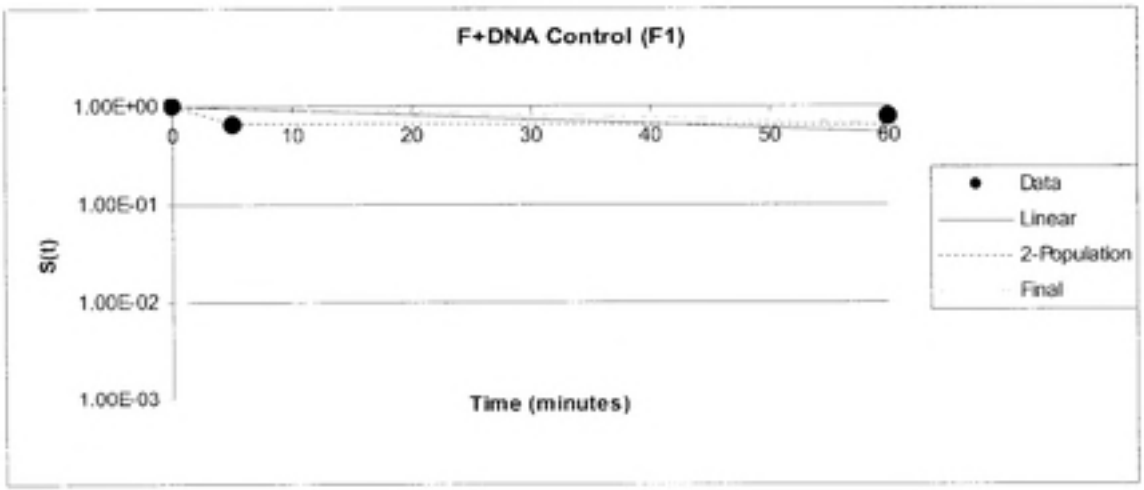

F+DNA Feed (10)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

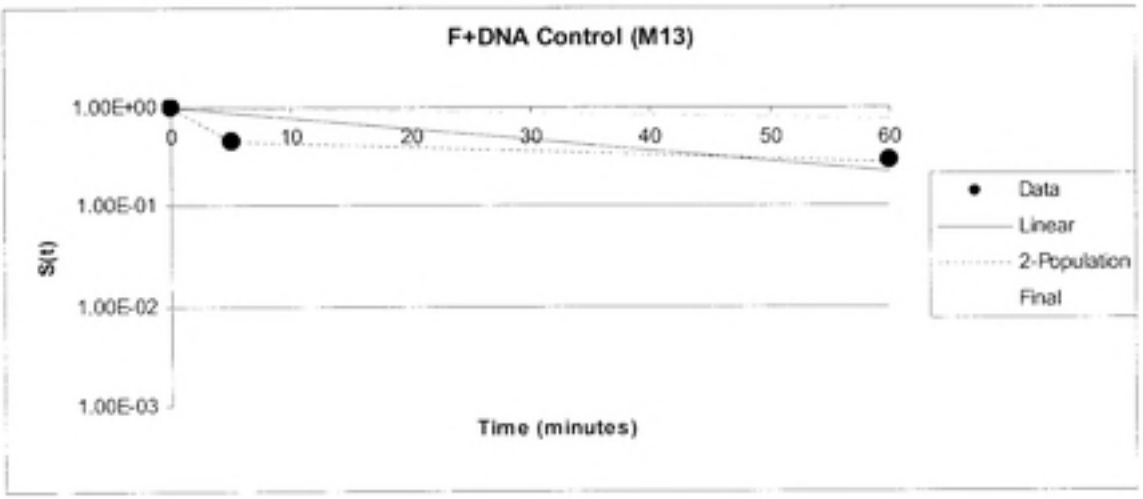

F+DNA Feed (11)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

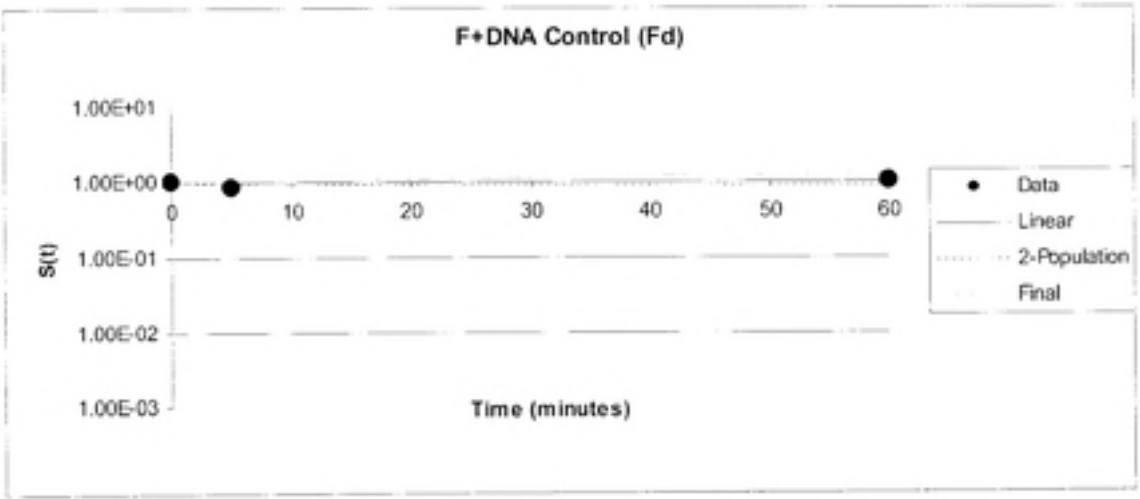

F+DNA Feed (12)
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final


F+DNA Feed (13)
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
Time (minutes)
Data
Linear
2-Population
Final


F+DNA Feed (14)
100E+00
100E-01
100E-02
100E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final


F+DNA Control (F1)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final


F+DNA Control (M13)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final


F+DNA Control (Fd)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
S(t)
0
10
20
30
40
50
60
Time (minutes)
Data
Linear
2-Population
Final

APPENDIX 7. Best-Fit Parameters for Inactivation of Male-Specific Coliphages at 53 °C

Group I Feed							
	1st Order	2 Population					
Isolate #	k	f1	k1	f2	k2	r^2 (5min)	r^2 (60min)
1a	0.32	0	0	1	0.32	0.852144	0.146607
1b	0.047	0.5	0.05	0.5	-	0.778801	0.0497871
2a	0.025	0.5	0.025	0.5	-	0.882497	0.2231302
2b	0.34	0.5	0.35	0.5	-	0.839457	0.1224564
3	0.77	0.5	0.77	0.5	-	0.680451	0.0098528
4	0.04	0.5	0.37	0.5	-	0.831104	0.1086091
5	0.09	0.9	0.25	0.1	0.04	0.339727	0.0090721
6	0.065	0.5	0.1	0.5	-	0.692666	0.0261329
7	0.07	0.5	0.075	0.5	-	0.687289	0.011109
8	0.08	0.5	0.5	0.5	0.063	0.405937	0.0114113

Group I Batch							
	1st Order	2 Population					
Isolate #	k	f1	k1	f2	k2	r^2 (5min)	r^2 (60min)
1a	0.03	0	0	1	-	0.847894	0.138069
1b	0.04	0.4	0.5	0.6	0.025	0.562332	0.133878
2	0.08	0.3	0.8	0.7	0.07	0.498776	0.010497
3a	0.07	0.4	0.5	0.6	0.053	0.493158	0.024951
3b	0.08	0.83	0.9	0.17	0.022	0.161512	0.045413
4	0.12	0.85	0.9	0.15	0.067	0.116743	0.002693
5	0.06	0.5	0.8	0.5	0.044	0.410417	0.035681
6	0.06	0.55	0.7	0.45	0.04	0.385037	0.040823
7	0.015	0	0.015	1	-	0.927743	0.40657
8	0.06	0	0	1	-	0.729789	0.022823
9	0.1	0.6	0.6	0.4	0.07	0.311747	0.005998
10	0.08	0.7	0.6	0.3	0.043	0.276813	0.022732
11	0.09	0.81	0.9	0.19	0.05	0.15697	0.00946
12	0.08	0.8	0.4	0.2	0.045	0.267971	0.013441
13	0.08	0.9	0.5	0.1	0.017	0.165728	0.036059

MS2 Control							
	1st Order	2 Population					
Isolate #	k	f1	k1	f2	k2	r^2 (5min)	r^2 (60min)
1	0.03	0.05	0.017	0.95	-	0.873716	0.1978987
2	0.077	0.01	0.077	0.99	-	0.680451	0.0098528
3	0.04	0.7	0.25	0.3	0.017	0.476107	0.1081787
4	0.03	0.7	0.07	0.3	0.005	0.785875	0.2327424
5	0.015	0	0	1	-	0.918512	0.3605949

* A dash (-) indicates that the reaction is first-order kinetics and the k_2 is negligible.

Group III Feed							
	1st Order	2 Population					
Isolate #	k	f1	k1	f2	k2	r^2 (5min)	r^2 (60min)
1	0.13	0.9	0.8	0.1	0.017	0.085213003	0.0011109
2a	0.07	0.5	0.12	0.5	-	0.65419188	0.018814877
2b	0.05	0.5	0.05	0.5	-	0.778800783	0.049787068
3	0.1	0.8	0.5	0.2	0.065	0.21017347	0.004048382
4	0.25	0.999	1.5	0.001	0.063	0.00128232	2.28227E-05
5	0.2	0.9	0.6	0.1	0.145	0.093240818	1.66586E-05
6	0.23	0.6	0.27	0.4	-	0.295519256	1.40409E-06
7	0.24	0.9	0.7	0.1	0.185	0.066830787	1.51123E-06
8	0.11	0.6	0.4	0.4	0.09	0.336252431	0.001806632
9	0.18	0.9	0.45	0.1	0.12	0.149740466	7.46586E-05
10	0.15	0.9	0.4	0.1	0.095	0.183990261	0.000334597

Qβ Controls							
	1st Order	2 Population					
Isolate #	k	f1	k1	f2	k2	r^2 (5min)	r^2 (60min)
1	0.19	0.04	0.1	0.96	0.9	0.034926	9.91501E-05
2	0.12	0	0	1	0.14	0.496585	0.000224867
3	0.18	0	0	1	0.19	0.386741	1.11955E-05
4	0.14	0.7	0.3	0.3	-	0.329276	0.000408121
5	0.32	0.9	0.9	0.1	0.19	0.048672	1.11955E-06
6	0.2	0.9	0.5	0.1	0.13	0.126081	4.09735E-05
7	0.15	0.9	0.5	0.1	0.09	0.137639	0.000451658
8	0.24	0.5	0.5	0.5	0.22	0.207478	9.25301E-07

* A dash (-) indicates that the reaction is first-order kinetics and the k_2 is negligible.

F+DNA Field Isolates							
	1st Order	2 Population					
Isolate #	k	f1	k1	f2	k2	r^2 (5min)	r^2 (60min)
1	0.77	0	0.5	1	0.075	0.687289	0.011109
2	0.009	0.1	0.05	0.9	-	0.955659	0.671715
3	0.002	0.01	0.001	0.99	-	0.995012	0.941765
4	0.002	0.01	0.001	0.99	-	0.975557	0.74341
5	0.009	0.2	0.5	0.8	0.003	0.804507	0.668216
6	0.009	0	0	1	-	1	1
7	0	0	0	1	-	1	1
8	0.04	0.7	0.2	0.3	0.009	0.544315	0.174829
9	0.1	0.9	0.5	0.1	0.043	0.154531	0.007577
10	0.03	0	0.03	1	-	0.860708	0.165299
11	0	0	0	1	-	0.97531	0.740818
12	0	0	0	1	-	1	1
13	0.015	0.1	0.015	0.9	-	0.927743	0.40657
14	0.002	0.01	0.002	0.99	-	0.99005	0.88692

F+DNA Controls							
	1st Order	2 Population					
Isolate #	k	f1	k1	f2	k2	r^2 (5min)	r^2 (60min)
F1	0.01	0.35	0.6	0.65	0	0.667425	0.65
M13	0.025	0.55	0.7	0.45	0.008	0.448964	0.278453
Fd	0.005	0.1	0.1	0.9	0	0.960653	0.900248

* A dash (-) indicates that the reaction is first-order kinetics and the k_2 is negligible.

APPENDIX 8. Comparison of Dilution Media in the Spot Titer Method

Media Comparison in Spot Titer Assay: Group I Field Isolate	
Media Used	Titer
3% Beef Extract	1.50E+08
Tryptic Soy Broth	1.80E+08
PBS (regular)	1.80E+08
PBS (Dubeccos)	1.80E+08

Media Comparison in Spot Titer Assay: Group I (MS2 ATCC) Isolate	
Media Used	Titer
3% Beef Extract	8.60E+09
Tryptic Soy Broth	6.00E+09
PBS (regular)	5.90E+09
PBS (Dubeccos)	3.00E+09

APPENDIX 9. Comparison of Spot Titer Assay and Double Agar Layer Method

Methods Comparison: Double Agar Layer (DAL) and Spot Titer Assay					
	Mean Titer DAL	Standard Deviation	Mean Titer SpotTiter	Standard Deviation	Z-Score
Trial 1	1.09E+03	3.62E+01	1.60E+03	4.37E+02	1.17
Trial 2	1.01E+03	7.06E+01	1.57E+03	2.45E+02	2.20
Trial 3	1.10E+03	8.40E+01	1.81E+03	4.56E+02	1.52

* There is a 95% probability that both methods give the same result in all trials.

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